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(54) Title: INHIBITORS OF CELL PROLIFERATION, THEIR PREPARATION AND USE

(57) Abstract

Disclosed is the characterization and purification of DNA encoding numerous polypeptides factors useful for the inhibition of cell (particularly, Schwann cell) proliferation. These factors are useful for the treatment of neural tumors. Also disclosed are the DNA sequences encoding novel polypeptides which may have use as agents which inhibit cell proliferation. Methods for the synthesis, purification, and testing of both known and novel polypeptides for their use as therapeutic and diagnostic aids in the treatment of diseases are also provided. Methods are also provided for the use of these polypeptides for the preparation of antibody probes. Such probes have diagnostic and therapeutic use in diseases involving neural and glial cells.

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INHIBITORS OF CELL PROLIFERATION, THEIR PREPARATION AND USE

Background of the Invention

The invention relates to compounds which are inhibitors of cell proliferation, having antiproliferative activity on a variety of cell types.

Many vertebrate cell types respond to diffusible growth factors as stimuli which regulate proliferation. A number of these growth factors and their cognate receptors have been purified, and the genes encoding them have been cloned and characterized (Sporn and Roberts eds. (1991) Peptide Growth Factors and their Receptors I and II. Springer-Verlaz, New York). Many cancers, which are diseases of cell proliferation, involve genetic modifications which affect the nature of the growth factor-receptor interaction. Such modifications can result in unregulated stimulation of proliferation in the receptor bearing target cell. Additionally, certain tumors of the nervous system involve the regulation of proliferation of cells from both the central and peripheral nervous systems.

The glial cells of vertebrates constitute the specialized connective tissue of the central and peripheral nervous systems. Important glial cells include the peripheral Schwann cells which provide both the metabolic support for neurons and the myelin sheathing around the axons of certain peripheral neurons, thereby forming individual nerve fibers. Schwann cells support neurons and provide a sheath effect by forming concentric layers of membrane around adjacent neuron axons, twisting as they develop around the axons. These myelin sheaths are a susceptible element of many nerve fibers. Damage to Schwann cells, or failure in growth and development, can be associated with significant demyelination or nerve degeneration characteristic of a number of peripheral nervous system diseases and disorders. In the development

of the nervous system, it has become apparent that cells require various factors to regulate their division and growth. Several regulators of Schwann cell proliferation and differentiation have been identified. Such factors play an important role in both the development and the regeneration (following injury) of the peripheral nervous system.

Brockes et al. ((1984) J. Neuroscience 4:75-83) describe a protein growth factor present in extracts from bovine brain and pituitary tissue, termed Glial Growth Factor (GGF). This factor stimulates cultured rat Schwann cells to divide against a background medium containing ten percent fetal calf serum. GGF has been described as having a molecular weight of 31 KD and readily forming dimers. Brockes ((1987) Meth. Enz. 147:217-225) describes a Schwann cell-based assay for 31 kD GGF and purification using reversed phase HPLC.

The J. Neuroscience article of Brockes et al., supra, describes methods of purification of GGF to apparent homogeneity. In brief, one large-scale purification method described involves extraction of the lyophilized bovine anterior lobes and chromatography of material obtained thereby, using NaCl gradient elution from CM cellulose. Gel filtration is then carried out with an Ultrogel column, followed by elution from a phosphocellulose column, and finally, small-scale SDS gel electrophoresis. Alternatively, the CM-cellulose material was applied directly to a phosphocellulose column, fractions from the column were pooled and purified by preparative native gel electrophoresis, followed by a final SDS gel electrophoresis.

Brockes et al. ((1980) J. Biol. Chem. 255:8374-8377) observe that in gel filtration experiments the major peak of growth factor activity is observed to migrate with a molecular weight of 56 KD, whereas in the first of the

above-described procedures activity was predominantly observed at molecular weight 31 KD. They report that the GGF dimer is largely removed as a result of the gradient elution from CM-cellulose in this procedure.

Benveniste et al. ((1985) PNAS 82:3930-3934) describe a T lymphocyte-derived glial growth promoting factor. This factor, under reducing conditions, exhibits a change in apparent molecular weight on SDS gels.

Kimura et al. ((1990) Nature 348:257-260) describe a factor they term Schwannoma-derived growth factor (SDGF) obtained from a sciatic nerve sheath tumor. The authors state that SDGF does not stimulate the incorporation of tritium-labelled TdR into cultured Schwann cells under conditions where, in contrast, partially purified pituitary fraction containing GGF is active. SDGF has an apparent molecular weight between 31 KD and 35 KD.

Davis et al. ((1990) J. Cell. Biol. 110:1353-1360) describe the screening of a number of candidate mitogens. The chosen candidate substances being examined for their ability to stimulate DNA synthesis in Rat Schwann cells in the presence of 10% FCS (fetal calf serum), with and without forskolin. One of the factors tested, GGF-carboxymethyl cellulose fraction (GGF-CM), was mitogenic in the presence of FCS, with and without forskolin. It was also observed that in the presence of forskolin platelet derived growth factor (PDGF) is a potent mitogen for Schwann cells. Previous to this finding, PDGF was not thought to have a mitogenic effect on Schwann cells.

Holmes et al. ((1992) Science 256:1205) and Wen et al. ((1992) Cell 69:559) demonstrate that DNA sequences which encode proteins which bind to a receptor ($p185^{erbB2}$) are associated with several human tumors.

The $p185^{erbB2}$ protein is a 185 kilodalton membrane spanning protein with tyrosine kinase activity. The

protein is encoded by the *erbB2* proto-oncogene (Yarden and Ullrich. (1988) Ann. Rev. Biochem. 57:443). The *erbB2* gene, also referred to as HER-2 (in human cells) and *neu* (in rat cells), is closely related to the receptor for epidermal growth factor (EGF). Recent evidence indicates that proteins which interact with (and activate the kinase of) $p185^{erbB2}$ induce proliferation in the cells bearing $p185^{erbB2}$ (Holmes et al. (1992) Science 256:1205; Dobashi et al. (1991) Proc. Natl. Acad. Sci. 88:8582; and Lupu et al. (1992) Proc. Natl. Acad. Sci. 89:2287).

Although ligands have been identified which stimulate proliferation of cells with certain receptors (e.g., the $p185^{erbB2}$ receptor), there exists a need to identify and isolate factors which act as inhibitors of cell proliferation at these receptor sites. Such inhibitors could be used for the purpose of treating cell proliferative disorders (e.g., neoplasms).

Summary of the Invention

In general, the invention provides methods for inhibiting proliferation of cells, including cells of the nervous system. The antiproliferative factors of the invention are alternative splicing products and fragments thereof of the DNA encoding the GGF/$p185^{erbB2}$ family of proteins.

The invention also provides a DNA sequence encoding a glial growth inhibitory factor; the sequence is included in the clone pGGF2HBS11 (ATCC Deposit No. 75347).

The peptide encoded by this clone is also a part of the invention. The invention further includes a peptide comprising a peptide encoded by the E sequence (SEQ ID Nos. 137 and 163) and at least a portion of the peptides encoded by brain derived DNA sequences flanking the E encoding sequences on clone pGGF2HBS11 (ATCC Deposit No. 75347). Preferably, the E encoded polypeptide sequence lacks 48

amino acids on the amino-terminal end and includes between 20 and 100 or, more preferably, between 25 and 70 amino acids of flanking the E encoded polypeptide. In addition, the E encoded polypeptide may be flanked by between 30 and 50, or, more preferably, between 35 and 45 amino acids on the carboxy terminal side of the E encoded segment. The sequences flanking the E encoded polypeptide are encoded by the DNA sequences flanking the E sequence present in clone pGGF2HBS11 (ATCC Deposit No. 75347).

Specifically, the invention also provides a method for inhibiting cell proliferation *in vitro* or *in vivo* comprising contacting the cell with

a) a polypeptide defined by the formula

VYBAZWX

wherein VYBAZWX is composed of the polypeptide segments shown in Figure 31 (SEQ ID Nos. 136-139, 141-147, 160, 161); wherein V comprises F, or is absent; wherein Y comprises polypeptide segment E, or is absent; wherein Z comprises polypeptide segment G or is absent; wherein W comprises C or is absent; and wherein X comprises polypeptide segments C/D HKL, C/D H, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, H, HK, HKL, or C/D C/D' D' HKL;

b) a polypeptide comprising FBA polypeptide segments having the amino acid sequences shown in Figure 31 (SEQ ID Nos. 136, 138, 139);

c) a polypeptide comprising FBA' polypeptide segments having the amino acid sequences shown in Figure 31 (SEQ ID Nos. 136, 138, 140, 168);

d) a polypeptide comprising FEBA polypeptide segments having the amino acid sequences shown in Figure 31 (SEQ ID Nos. 136-139); or

e) a polypeptide comprising FEBA' polypeptid segments having the amino acid sequences corresponding to polypeptide segments shown in Figure 31 (SEQ ID Nos. 136-138, 140, 168);

f) a polypeptide comprising EBA' polypeptide segments having the amino acid sequences corresponding to polypeptide segments shown in Figure 31 (SEQ ID Nos. 136, 138, 140, 168); or

g) a polypeptide comprising a portion of the E sequence (SEQ ID Nos. 137 and 163) and flanked by new sequence not contained in F, B, A, C/D, C/D', D, D', HK or L and contained in clone pGGF2HBS11, ATCC Deposit No. 75347; or to glial cells (i.e., astrocytes and microglial cells of the central and peripheral nervous system and Schwann cells of the peripheral nervous system).

The invention also provides a method of inhibiting proliferation of cells, including cells of the nervous system, by a method which consists of contacting the cells with a compound which specifically binds the $p185^{erbB2}$ receptor of the cell type.

Also included is the method comprising the administration of any of the above mentioned peptides when the peptides are administered in the treatment or prophylaxis of a nervous disease or disorder. Further included in the invention is the method of administering any of the above mentioned peptides when the cell is present in a mammal and the contacting of the cell is carried out by the administration of the peptide to the mammal for the prophylaxis or treatment of a pathophysiological condition in the mammal which involves the stated cell. Also included is the use of the method, as stated above, wherein the condition involves a disease of cell proliferation, such as a tumor, and more specifically, where the condition involves peripheral nerve

damage caused by a tumor of the nervous system. Also a part of the invention is the administration of the inhibitory factors for the purpose of increasing myelination of existing or regenerated neural tissue.

Further included as a part of the invention are methods comprising administration of any of the above mentioned polypeptides to a cell when the cell is present in a mammal and the contacting of the cell is carried out by administering the peptide to the mammal for the prophylaxis or treatment of a condition which involves one of the following conditions: a tumor of the Schwann cells, for example, neurofibromatosis, malignant Schwannomas or neurofibrosarcomas; a meningioma; a bilateral acoustic neuroma; an astrocytoma; a retinoblastoma; a neuroglioma; a neuroblastoma; an adenocarcinoma; or a glioma, by the method comprising administering to the mammal an effective amount of a polypeptide, as defined above.

The invention also includes a method for producing an antibody specific for a polypeptide, consisting of immunizing a mammal with a polypeptide selected from the above listed polypeptides, or a fragment thereof, and purifying the antibody from the tissue of the animal, or from a hybridoma made using the tissue.

Furthermore, the invention provides a method for detecting, in a sample, the presence of a molecule capable of binding to a receptor which binds to a polypeptide selected from the above mentioned polypeptides, and contacting the sample with the polypeptide together with the receptor, and detecting inhibition of the binding of the polypeptide to the receptor as an indication of the presence of a receptor binding molecule in the sample. The invention also provides methods for determining whether such a competitive inhibitor is an antagonist or agonist of receptor function.

Thus, factors useful in the methods of the invention

are:-

(a) basic polypeptide factors having antiproliferative activity when contacted with cells, including cells of the nervous system and specifically Schwann cells, and containing within their amino acid sequences one or more of the following peptide sequences:

F K G D A H T E
A S L A D E Y E Y M X K
T E T S S S G L X L K
A S L A D E Y E Y M R K
A G Y F A E X A R
T T E M A S E Q G A
A K E A L A A L K
F V L Q A K K
E T Q P D P G Q I L K K V P M V I G A Y T
E Y K C L K F K W F K K A T V M
E X K F Y V P
K L E F L X A K; and

(b) basic polypeptide factors capable of inhibiting the division of cells, including cells of the nervous system and particularly Schwann cells, and containing within their amino acid sequences, respectively, one or more of the following peptide sequences:

V H Q V W A A K
Y I F F M E P E A X S S G
L G A W G P A F P V X Y
W F V V I E G K
A S P V S V G S V Q E L Q R
V C L L T V A A L P P T
K V H Q V W A A K
K A S L A D S G E Y M X K
D L L L X V

The peptide sequences set out above, derived from lower and higher molecular weight polypeptide factors described in detail below, are also aspects of this

invention in their own right. These sequences are potentially useful as therapeutics, probes for large polypeptide factors, for investigating, isolating or preparing such factors (or corresponding gene sequences) from a range of different species, or preparing such factors by recombinant technology, and in the generation of antibodies (preferably monoclonal), by conventional technologies, which are themselves useful as investigative tools and potential medicaments. Such antibodies are included within this invention. The invention also includes inhibitors of cell proliferation encoded by gene sequences obtainable using the peptide sequences of the invention.

The invention further includes methods for use of a polypeptide factor having cell, including cells of the nervous system, antiproliferative activity and including an amino acid sequence encoded by:

(a) a DNA sequence shown in any one of Figures 28a, 28b or 28c (SEQ ID Nos. 133-135 respectively);

(b) a DNA sequence shown in Figure 22 (SEQ ID No. 89);

(c) the DNA sequence represented by nucleotides 281-557 of the sequence shown in Figure 28a (SEQ ID No. 133); or

(d) a DNA sequence hybridizable to any one of the DNA sequences according to (a), (b) or (c).

While the present invention is not limited to a particular set of hybridization conditions, the following protocol gives general guidance which may, if desired, be followed:

Thus, DNA probes may be labelled to high specific activity (approximately 10^8 to 10^9 dpm ^{32}P per μg) by nick-translation or by PCR reactions according to Schowalter and Sommer ((1989) Anal. Biochem. 177:90-94) and

purified by desalting on G-150 Sephadex columns. Probes may be denatured (10 minutes in boiling water followed by immersion into ice water), then added to hybridization solutions of 80% buffer B (2g polyvinylpyrolidine, 2g Ficoll-400, 2g bovine serum albumin, 50μl 1M Tris HCL (pH 7.5), 58g NaCl, 1g sodium pyrophosphate, 10g sodium dodecyl sulfate, 950μl H_2O) containing 10% dextran sulfate at 10^6 dpm ^{32}P per μl and incubated overnight (say, 16 hours) at 60°C. The filters may then be washed at 60°C, first in buffer B for 15 minutes followed by three 20-minute washes in 2X SSC, 0.1% SDS then one for 20 minutes in 1x SSC, 0.1% SDS.

The methods of the invention take advantage of the fact that a Glial Growth Factor and the $p185^{erbB2}$ ligand protein are encoded by the same gene. A variety of messenger RNA splicing variants (and their resultant proteins) are derived from this gene, and many of these products exhibit $p185^{erbB2}$ binding. This binding may result in either cell proliferation or cessation of cell division. At least two of the gene products (GGFI and GGFII) have been used to induce Schwann cell mitogenic activity. This invention employs some of the known products of the GGF/$p185^{erbB2}$ ligand gene (described in the references listed above) as inhibitors of cell proliferation and, more specifically, as inhibitors of glial cell proliferation.

This invention also relates to other, not yet naturally isolated splicing variants of the Glial Growth Factor gene. Figure 30 shows the known patterns of splicing derived from polymerase chain reaction experiments (on reverse transcribed RNA) and analysis of cDNA clones (as presented within) and derived from what has been published as sequences encoding $p185^{erbB2}$ ligands (Peles et al. (1992) Cell 69:205 and Wen et al. (1992) Cell 69:559). These patterns, as well as additional patterns disclosed herein, represent probable existing splicing variants.

Thus other aspects of the invention are :

Methods for the use of a series of human and bovine polypeptide factors having cell antiproliferative activity, including the inhibition of the division of cells of the nervous system, such as Schwann cells. Such peptide sequences are shown in Figure 31-34, (SEQ ID Nos. 136-137), respectively.

The human peptide sequences described above and presented in Figures 31-34, SEQ ID Nos. 136-137 respectively, represent a series of splicing variants which can be isolated as full length complementary DNA's (cDNA's) from natural sources (cDNA libraries prepared from the appropriate tissues) or assembled as DNA constructs with individual exons (e.g., derived as separate exons) by one skilled in the art.

Other compounds, in particular, peptides, which bind specifically to the $p185^{erbB2}$ receptor can also be used according to the invention as inhibitors of glial cell proliferation. A candidate compound can be routinely screened for $p185^{erbB2}$ binding, and, if it binds, can be screened for inhibition of cell proliferation using the methods described herein.

The invention includes the use of any modifications or equivalents of the above polypeptide factors which do not exhibit a significant reduction in the stated inhibitory activity. For example, modifications in which amino acid content or sequence is altered without substantially adversely affecting inhibitory activity are included. By way of illustration, in EP-A 109748 muteins of native proteins are disclosed in which the possibility of unwanted disulfide binding is avoided by replacing any cysteine in the native sequence which is not necessary for biological activity with a neutral amino acid. The statements of effect and use contained herein are therefore to be construed accordingly, with such uses and effects

employing modified or equivalent factors as aforesaid being part of the invention.

The peptides useful in the invention can be made recombinantly using DNA constructs comprising DNA sequences, as defined above, in operable reading frame position in vectors under the control of control sequences so as to permit expression of the sequences in suitable host cells after transformation thereof by said constructs (preferably the control sequence includes a regulatable promoter, e.g. Trp) - it will be appreciated that the selection of a promoter and regulatory sequences (if any) are matters of choice for those of ordinary skill in the art.

The factors of the invention can be formulated for pharmaceutical or veterinary use by combination with an acceptable diluent, carrier or excipient and/or in unit dosage form. In using the factors of the invention, conventional pharmaceutical or veterinary practice may be employed to provide suitable formulations or compositions.

Thus, the formulations of this invention can be applied to parenteral administration, for example, intravenous, subcutaneous, intramuscular, intraorbital, opthalmic, intraventricular, intracranial, intracapsular, intraspinal, intracisternal, intraperitoneal, topical, intranasal, aerosol, scarification, and also oral, buccal, rectal or vaginal administration.

The formulations of the invention may also be administered by the transplantation into the patient of host cells expressing the DNA of the instant invention or by the use of surgical implants which release the formulations of the invention.

Parenteral formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal

drops, or aerosols.

Methods well known in the art for making formulations are to be found in, for example, "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain as excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes, biocompatible, biodegradable lactide polymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the present factors. Other potentially useful parenteral delivery systems for the factors include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration, methoxysalicylate for rectal administration, or citric acid for vaginal administration.

The present factors can be used as the sole active agents or can be used in combination with other active ingredients.

The concentration of the present factors in the formulations of the invention will vary depending upon a number of issues, including the dosage to be administered, and the route of administration.

In general terms, the factors of this invention may be provided in an aqueous physiological buffer solution containing about 0.1 to 10% w/v compound for parenteral administration. General dose ranges are from about 1 μg/kg to about 1 g/kg of body weight per day; a preferred dose

range is from about 0.01 mg/kg to 100 mg/kg of body weight per day. The preferred dosage to be administered is likely to depend upon the type and extent of progression of the pathophysiological condition being addressed, the overall health of the patient, the make up of the formulation, and the route of administration.

As indicated above, cell proliferation, particularly that of Schwann cells (the glial cells of the peripheral nervous system) and other cells of the nervous system is inhibited in the presence of the factors of the invention.

There are a variety of tumors of glial cells, the most common of which is probably neurofibromatosis, which is a patchy small tumor created by overgrowth of glial cells. Also, it has been found that an activity very much like GGF can be found in some Schwann cell tumors (Brockes et al., Ann. Neurol. 20:317 (1986)). Therefore inhibitors of GGF action on their receptors provides a therapy of a glial tumor. This therapy comprises administering an effective amount of a substance which inhibits the binding of a stimulatory factor as defined above to its receptor. Additionally, given the association of GGF receptor amplification with human adenocarcinomas (Kraus et al., (1987) EMBO J. 6:605; Slamon et al. (1987) Science 235:177; Varley et al. (1987) Oncogene 1:423; and van de Vijver et al. (1987) Mol Cell Biol 7:2019) and tumors of the breast and ovary (Slamon et al. supra; Varley et al. supra: Venter et al. (1987) Lancet ii:67; Zhou et al. (1987) Cancer Res. 47:6123; Berger et al. (1988) Cancer Res. 48:1238; Tsuda et al. (1989) Cancer Res. 49:3104; Slamon et al. (1989) Science 244:707), a similar therapeutic approach may be taken with adenocarcinomas and tumors of breast and ovarian tissues.

In general, the invention includes the use of present polypeptide factors in the prophylaxis or treatment of any pathophysiological condition in which a

factor-sensitive or factor-responsive cell type is involved.

The polypeptide factors of the invention can also be used as immunogens for making antibodies, such as monoclonal antibodies, following standard techniques. Such antibodies are included within the present invention. These antibodies can be used for therapeutic or diagnostic purposes. Thus, conditions associated with abnormal levels of the factor may be tracked by using such antibodies. In vitro techniques can be used, employing assays on isolated samples using standard methods. Imaging methods can also be employed in which the antibodies are, for example, tagged with radioactive isotopes which can be remotely imaged from outside the body using techniques employed in the art of, for example, tumour imaging.

Such antibodies, as described above, may also be used for therapeutic purposes. Anti-idiotypic antibodies raised against the polypeptide factors of the invention or idiotypic antibodies raised against their cognate receptor can be used as antagonists of GGF/*erbB2* ligand induced proliferation of $p185^{erbB2}$ bearing cells.

The invention also includes the general use of the present factors as inhibitors of cell proliferation in vivo or in vitro, and the methods for such use. One embodiment is thus a method for producing a tumor cell antiproliferative effect in a vertebrate by administering an effective amount of a factor of the invention. An example of such a method is the treatment or prophylaxis of nervous system tumors or tumors of other tissues.

A further general aspect of the invention is the use of a factor of the invention in the manufacture of a medicament, preferably for the treatment of a nervous disease or disorder.

Also included in the invention are the use of the factors of the invention in competitive assays to identify

or quantify molecules having receptor binding characteristics corresponding to those of said polypeptides. The polypeptides may be labelled, optionally with a radioisotope and these labelled products may be used to determine if receptor binding exists. A competition assay can identify both antagonists and agonists of the relevant receptor. Any competition for receptor binding between a known agonist and an antagonist (shown to bind the receptor) in a bioassay would be reflected by a decrease in biological activity with in an increase in concentration of antagonist.

In another aspect, the invention provides the use of the factors in an affinity isolation process, e.g., affinity chromatography, for the separation of a respective corresponding receptor. Such processes for the isolation of receptors corresponding to particular proteins are known in the art, and a number of techniques are available and can be applied to the factors of the present invention. For example, in relation to IL-6 and IFN-gamma, the reader is referred to Novick et al. ((1990) J. Chromatogr. 510:331-7), in relation to gonadotropin releasing hormone, reference is made to Hazum ((1990) J. Chromatogr. 510:233-8), in relation to G-CSF, reference is made to Fukunaga et al. ((1990) J. Biol. Chem. 265:13386-13390), in relation to IL-2, reference is made to Smart et al. ((1990) J. Invest. Dermatol. 94:158S-163S), and in relation to human IFN-gamma, reference is made to Stefanos et al. ((1989) J. Interferon Res. 9:719-30)

The following examples are not intended to limit the invention, but are intended to usefully illustrate the same, and provide specific guidance for effective preparative techniques. Examples 1-4 teach the purification and consequent cloning of bovine DNA sequences encoding GGF Examples 5 and 7 demonstrate the isolation of human DNA sequences encoding GGF. Examples 8 and 9

demonstrate the isolation of splicing variants. Examples 10 and 11 show specific antiproliferative variants and examples of their function. Examples 12 and 13 demonstrate the production and testing of antiproliferative molecules.

Brief Description of the Drawings

The drawings will first be described.

Drawings

Figures 1 to 8 relate to Example 1 hereinafter, and are briefly described below:

Figure 1 is the profile for product from carboxymethyl cellulose chromatography;

Figure 2 is the profile for product from hydroxylapatite HPLC;

Figure 3 is the profile for product from Mono S FPLC;

Figure 4 is the profile for product from Gel filtration FPLC;

Figures 5 and 6 depict the profiles for the two partially purified polypeptide products from reversed-phase HPLC; and

Figures 7 and 8 depict dose response curves for the GGF-I and GGF-II fractions from reversed-phase HPLC using either a fetal calf serum or a fetal calf plasma background;

Figures 9 to 12 depict peptides derived from GGF-I and GGF-II, (SEQ ID Nos. 1-53 and 169) (see Example 2, hereinafter), Figures 10 and 12 specifically depict novel sequences:

In Figure 10, Panel A, the sequences of GGF-I peptides used to design degenerate oligonucleotide probes and degenerate PCR primers are listed (SEQ ID Nos. 20-30). Some of those sequences in Panel A were also used to design synthetic peptides. Panel B shows the novel peptides that were too short (less than 6 amino acids) for the design of

degenerate probes or degenerate PCR primers (SEQ ID Nos. 31 and 52);

In Figure 12, Panel A, the sequences of GGF-II peptides used to design degenerate oligonucleotide probes and degenerate PCR primers are listed (SEQ ID Nos. 45-52). Some of these sequences in Panel A were also used to design synthetic peptides. Panel B shows the novel peptides that were too short (less than 6 amino acids) for the design of degenerate probes or degenerate PCR primers (SEQ ID No. 53);

Figures 13 to 20 relate to Example 3 hereinafter, and show various aspects of the mitogenic activity of factors related to the invention;

Figures 21 to 28 (a, b and c) relate to Example 4 hereinafter, and are briefly described below:

Figure 21 lists the degenerate oligonucleotide probes (SEQ ID Nos. 54-88) designed from the novel peptide sequences listed in Figure 10, Panel A and Figure 12, Panel A;

Figure 22 (SEQ ID No. 89) depicts a stretch of the putative bovine GGF-II gene sequence from the recombinant bovine genomic phage GGF2BG1, which contains the binding site of degenerate oliognucleotide probes 609 and 650 (see Figure 21, SEQ ID Nos. 69 and 72, respectively).

Shown are the coding strand of the DNA sequence and the deduced amino acid sequence in the third reading frame. The sequence of peptide 12 from GGF-2 (shown in bold) is part of a 66 amino acid open reading frame (nucleotides 75272);

Figure 23 lists the degenerate PCR primers (Panel A, SEQ ID No. 90-108) and unique PCR primers (Panel B, SEQ ID Nos. 109-119) used in experiments to isolate segments of the bovine GGF-II coding sequences present in RNA from posterior pituitary;

Figure 24 summarizes the nine distinct contiguous bovine GGF-II cDNA structures and sequences that were obtained in PCR amplification experiments using the list of primers in Figure 7, Panels A and B, on RNA from posterior pituitary. The top line of the Figure shows a schematic of the exon sequences which contribute to the cDNA structures that were characterized;

Figure 25 is a physical map of bovine recombinant phage GGF2BG1. The bovine DNA fragment is roughly 20 kb in length and contains two exons (bold) of the bovine GGFII gene. Restriction sites for the enzymes Xbal, Spe I, Ndel, EcoRI, Kpnl, and SstI have been placed on this physical map. Shaded portions correspond to fragments which were subcloned for sequencing;

Figure 26 shows schematically the structure of three alternative gene products of the putative bovine GGF-II gene. Exons are listed A through E in the order of their discovery. The alternative splicing patterns 1, 2 and 3 generate three overlapping deduced protein structures (GGF2BPP1, 2, and 3), which are displayed in the various Figures 28;

Figure 27 compares the GGF-I and GGF-II sequences identified in the deduced protein sequences (SEQ ID Nos. 120-132) shown in Figures 28a, 28b and 28c with the novel peptide sequences listed in Figures 10 and 12. The Figure shows that six of the nine novel GGF-II peptide sequences are accounted for in these deduced protein sequences. Two peptide sequences similar to GGF-I sequences are also found;

Figure 28a shows the coding strand DNA sequence and deduced amino acid sequence of the cDNA (SEQ ID No. 133) obtained from splicing pattern number 1 shown in Figure 26. This partial cDNA of the putative bovine GGF-II gene encodes a protein of 206 amino acids in length. Peptides shown in bold were those identified from the lists

presented in Figures 10 and 12. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA);

Figure 28b shows the coding strand DNA sequence and deduced amino acid sequence of the cDNA (SEQ ID No. 134) obtained from splicing pattern number 2 shown in Figure 26. This partial cDNA of the putative bovine GGF-II gene encodes a protein of 281 amino acids in length. Peptides shown in bold were those identified from the lists presented in Figures 10 and 12. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA);

Figure 28c shows the coding strand DNA sequence and deduced amino acid sequence of the cDNA (SEQ ID No. 135) obtained from splicing pattern number 3 shown in Figure 26. This partial cDNA of the putative bovine GGF-II gene encodes a protein of 257 amino acids in length. Peptides shown in bold were those identified from the lists presented in Figures 10 and 12. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA); and the DNA sequences shown in Figures 28a, 28b and 28c are themselves further aspects of this invention; and the invention further includes polypeptides encoded by said sequences;

Figure 29 relates to Example 7, hereinafter, and shows an autoradiogram of a cross hybridization analysis of putative bovine GGF-II gene sequences to a variety of mammalian DNAs on a Southern blot. The filter contains lanes of Eco RI-digested DNA (5 Mg per lane) from the species listed in the Figure. The probe detects a single strong band in each DNA sample, including a four kb fragment in the bovine DNA as anticipated by the physical map in Figure 25. Bands of relatively minor intensity are observed as well, which could represent related DNA sequences. The strong hybridizing band from each of the

other mammalian DNA samples presumably represents the GGF-II homologue of those species.

In Example 1 hereinafter, unless otherwise indicated, all operations were conducted at 40°C, and, with reference to Figures 1 to 6, activity at each stage was determined using the Brockes (Meth. Enz., supra) techniques with the following modifications. Thus, in preparing Schwann cells, 5μM forskolin was added in addition to DMEM (Dulbecco's modified Eagle's medium), FCS and GGF. Cells used in the assay were fibroblast-free Schwann cells at passage number less than 10, and these cells were removed from flasks with trypsin and plated into flat-bottomed 96-well plates at 3.3 thousand cells per microwell.

^{125}IIUdR was added for the final 24 hours after the test solution addition. The background (unstimulated) incorporation to each assay was less than 100 cpm, and maximal incorporation was 20 to 200 fold over background depending on Schwann cell batch and passage number.

In the case of the GGF-I and GGF-II fractions from reversed-phase HPLC as described below in Example 1, two dose response curves were also produced for each factor, using exactly the above method for one of the curves for each factor, and the above method modified in the assay procedure only by substituting fetal calf plasma for fetal calf serum to obtain the other curve for each factor. The results are in Figures 7 and 8.

Figure 30 is a schematic diagram of representative splicing variants. The coding segments are represented by F, E, B, A, G, C, C/D, C/D', D, D', H, K and L. The location of the peptide sequences derived from purified protein are indicated by "o".

Figure 31 (SEQ ID No. 136-147, 160, 161 and 168) (is a listing of the DNA sequences and predicted peptide sequences of the coding segments of GGF. Line 1 represents the predicted amino acid sequence of bovine GGF, line 2

represents the nucleotide sequence of bovine GGF, line 3 represents the nucleotide sequence of human GGF (heregulin) (nucleotide base matches are indicated with a vertical line) and line 4 represents the predicted amino acid sequence of human GGF/heregulin *where it differs from the predicted bovine sequence*. Coding segment K represents only the bovine sequence. The human and bovine coding segments for both E and A' are provided. Coding segment D' represents only the human (heregulin) sequence.

Figure 32 is the predicted GGF2 amino acid sequence and nucleotide sequence of BPP5 (SEQ ID No. 148). The upper line represents the nucleotide sequence and the lower line represents the predicted amino acid sequence.

Figure 33 is the predicted amino acid sequence and nucleotide sequence of GGF2BPP2 (SEQ ID No. 149). The upper line represents the nucleotide sequence and the lower line represents the predicted amino acid sequence.

Figure 34 is the predicted amino acid sequence and nucleotide sequence of GGF2BPP4. The upper line represents the nucleotide sequence and the lower line represents the predicted amino acid sequence.

Figure 35 (SEQ ID Nos. 151-152) depicts the alignment of two GGF peptide sequences (GGF2bpp4 and GGF2bpp5) with the human EGF (hEGF) peptides sequences. Asterisks indicate positions of conserved cysteines.

Figure 36 depicts the level of GGF activity (Schwann cell mitogenic assay) and tyrosine phosphorylation of a ca. 200kD protein (intensity of a 200 kD band on an autoradiogram of a Western blot developed with an antiphosphotyrosine polyclonal antibody) in response to increasing amounts of GGF.

Figure 37 is a list of splicing variants derived from the sequences shown in Figure 31.

Figure 38 is a scale coding segment map of the clone. T3 refers to the bacteriophage promoter used to

produce mRNA from the clone. R = flanking Ec RI restriction enzyme sites. 5′ UT refers to the 5′ untranslated region. E, B, A, C, C/D′, and D refer to the coding segments. O = the translation start site. Λ = the 5′ limit of the region homologous to the bovine E segment (see example 6) and 3′ UT refers to the 3′ untranslated region.

Figure 39 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL1 (SEQ ID No. 154).

Figure 40 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL2 (SEQ ID No. 155).

Figure 41 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL3 (SEQ ID No. 156).

Figure 42 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL4 (SEQ ID No. 157).

Figure 43 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL5 (SEQ ID No. 158).

Figure 44 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL6 (SEQ ID No. 159).

Figure 45 is the predicted amino acid sequence (middle) and nucleic sequence (top) of GGF2HBS5 (SEQ ID No. 167). The bottom (intermittent sequence represents peptide sequences derived from GGFII preparations (see Figures 11, 12).

Detailed Description

The invention pertains to methods for the use of novel factors which are inhibitors of cell, particularly neural and glial cell proliferation, and the use of DNA

sequences encoding these factors. Disclosed are several gene splicing variants of these factors which may encode inhibitors of cell division.

Holmes et al. ((1992) Science 256:1205) and Wen et al. ((1992) Cell 69:559) demonstrate that DNA sequences encoding proteins which bind to a receptor associated with several human tumors ($p185^{erbB2}$) share a great deal of homology with GGF DNA sequences. This provides evidence to indicate that the bovine GGFs and the human and rat $p185^{erbB2}$ ligands are encoded by the same (homologous) gene and that ligand groups both interact with the same receptor ($p185^{erbB2}$).

The $p185^{erbB2}$ protein is a 185 kilodalton membrane spanning protein with tyrosine kinase activity. The protein is encoded by the *erbB2* proto-oncogene (Yarden and Ullrich. (1988) Ann. Rev. Biochem. 57:443). The *erbB2* gene, also referred to as HER-2 (in human cells) and *neu* (in rat cells), is closely related to the receptor for epidermal growth factor (EGF). Recent evidence indicates that proteins which interact with (and activate the kinase of) $p185^{erbB2}$ induce proliferation in the cells bearing $p185^{erbB2}$ (Holmes et al. (1992) Science 256:1205; Dobashi et al. (1991) Proc. Natl. Acad. Sci. 88:8582; Lupu et al. (1992) Proc. Natl. Acad. Sci. 89:2287). This evidence supports the conclusion that the gene encoding GGF's and the $p185^{erbB2}$ binding proteins are responsible for the production of a family of growth factors which have pleiotrophic effects in that they target both neural cells, particularly Schwann cells, and cells which give rise to human adenocarcinoma and other carcinomas.

Furthermore, it is evident that the gene encoding GGF and $p185^{erbB2}$ binding proteins produces a number of variably-sized, differentially-spliced RNA transcripts that give rise to a series of proteins, which are of different lengths and contain some common peptide sequences and some

unique peptide sequences. This is supported by the evidence that differentially spliced sequences are recoverable from bovine posterior pituitary RNA (as presented herein), and human breast cancer cell line (MDA-MB-231) RNA (Holmes et al. (1992) Science 256:1205). Further support for this "one gene: multiple product" conclusion derives from the wide size range of proteins which act as both mitogens for Schwann cells (as disclosed herein) and ligands for the $p185^{erbB2}$ receptor (see below).

Further evidence to support the fact that the genes encoding GGF and $p185^{erbB2}$ receptor ligands are homologous comes from nucleotide sequence comparison. Holmes et al. ((1992) Science, 256:1205-1210) demonstrate the purification of a 45-kilodalton human protein (heregulin) which specifically interacts with the $p185^{erbB2}$ receptor. The predicted sequences of the polypeptides encoded by these human DNA sequences match very closely with the sequences predicted from the Glial Growth Factor sequences. Peles et al. ((1992) Cell 69:205) and Wen et al ((1992) Cell 69:559) describe a complementary DNA isolated from rat cells encoding a protein called *neu* differentiation factor (NDF), which shares homology with the heregulin sequences described by Holmes et al. In addition, the translation product of the NDF cDNA has $p185^{erbB2}$ binding activity. Several other groups have reported the purification of proteins of various molecular weights with *erbB2* binding activity. These groups include Lupu et al. ((1992) Proc. Natl. Acad. Sci. USA 89:2287), Yarden and Peles ((1991) Biochemistry 30:3543), Lupu et al ((1990) Science 249:1552), and Dobashi et al. ((1991) Biochem Biophys. Res. Comm. 179:1536).

It has been established that the $p185^{erbB2}$ oncogene and, by inference, its cognate ligands play a significant role in the development and maintenance of several types of tumors. Amplification and overexpression of *erbB2* has been

associated with human adenocarcinomas from several tissues (Kraus et al. (1987) EMBO J. 6:605; Slamon et al. (1987) Science 235:177; Varley et al. (1987) Oncogene 1:423; and van de Vijver et al. (1987) Mol Cell Biol 7:2019). An association has also been reported with breast and ovarian cancer (Slamon et al. supra; Varley et al. supra: Venter et al. (1987) Lancet ii:67; Zhou et al. (1987) Cancer Res. 47:6123; Berger et al. (1988) Cancer Res. 48:1238; Tsuda et al. (1989) Cancer Res. 49:3104; Slamon et al. (1989) Science 244:707).

There is also evidence that the *erbB2* gene plays a role in oncogenesis of cells of the Schwann cell lineage (Perantoni et al. (1987) Proc. Nat. Acad. Sci. 84:6317; Nikitin et al. (1991) Proc. Nat. Acad. Sci. 88:9939). Several tumor types are a result of abnormal proliferation of Schwann cells and these include neurofibromas, and malignant schwannomas and neurofibrosarcomas.

As candidate ligands for the *erbB2* receptor, the GGFs could play a significant role in the development of the tumors described above.

As outlined above, the gene encoding the GGFs and the $p185^{erbB2}$ ligands gives rise to a number of variant transcripts which encode a variety of proteins. Several of these variant proteins bind to the $p185^{erbB2}$ receptor on neural cells, including Schwann cells (described above and disclosed herein), as well as to the same receptor on tumor cell lines as described above. Some of these variant proteins activate cell proliferation in Schwann cells and in tumor cell lines (described above and disclosed herein). Other variants may possibly interfere with the activity of the ligands which stimulate proliferation by competing with those ligands for binding sites on the $p185^{erbB2}$ receptor. Chan et al. ((1991) Science 254:1382) showed that a naturally occurring hepatocyte growth factor (HGF) variant was derived from a smaller transcript encoded by

the same gene as the full length molecule. The truncated protein encoded by the variant transcript specifically inhibits HGF induced mitogenesis and was demonstrated to compete with HGF for binding to the HGF receptor. The HGF receptor has been identified as the *c-met* proto-oncogene product. Thus, these variant versions of growth factor proteins may play a significant regulatory role in the control of cell proliferation. GGF-related factors which inhibit glial proliferation will be therapeutically useful as anti-proliferative compounds for the treatment of tumors of the neural system.

It has been shown that myelination by Schwann cells and oligodendrocytes is regulated by the proliferative state (Jessen et al., 1991 *Ann NY Acad Science* 633:78-89). When the cell withdraws from the proliferative cycle the myelination process appears to begin. Factors of the invention which induce Schwann cells and oligodendrocytes to exit the proliferative cell cycle and enter the quiescent state may be administered to increase myelination of existing or newly regenerated neural tissue in a mammal suffering from diseases or disorders of demyelination. Examples of diseases and disorders which may be treated using an inhibitor of mutagenesis include Charot-Marie-Tooth disease (particularly type I and type III), peroneal muscular atrophy, Dejérine-Sottos disease (type III hereditary motor and sensory neuropathy), multiple sclerosis, chronic inflammatory demyelinating polyradiculoneuropathy, chronic liver disease, diphtheritic polyneuritis, Guillain-Barré syndrome, hypothyroid polyneuropathy, metachromatic leukodystrophy, type I hereditary motor and sensory neuropathy, type III hereditary motor and sensory neuropathy, and vasculitic neuropathy.

EXAMPLE 1

I. Preparation of Factor-CM Fraction

4,000 frozen whole bovine pituitaries (c.a. 12 kg) were thawed overnight, washed briefly with water and then homogenized in an equal volume of 0.15 M ammonium sulphate in batches in a Waring Blender. The homogenate was taken to pH 4.5 with 1.0 M HCl and centrifuged at 4,900g for 80 minutes. Any fatty material in the supernatant was removed by passing it through glass wool. After taking the pH of the supernatant to 6.5 using 1.0 M NaOH, solid ammonium sulphate was added to give a 36% saturated solution. After several hours stirring, the suspension was centrifuged at 4,900 g for 80 minutes and the precipitate discarded. After filtration through glass wool, further solid ammonium sulphate was added to the supernatant to give a 75% saturated solution which was once again centrifuged at 4,900 g for 80 minutes after several hours stirring. The pellet was resuspended in c.a. 2 L of 0.1 M sodium phosphate pH 6.0 and dialyzed 3 x 40 L of the same buffer. After confirming that the conductivity of the dialysate was below 20.0 μSiemens, it was loaded onto a Bioprocess column (120 x 113 mm, Pharmacia) packed with carboxymethyl cellulose (CM-52, Whatman) at a flow rate of 2 $\mu l.min^{-1}$ The column was washed with 2 volumes of 0.1 M sodium phosphate pH 6.0, followed by 2 volumes of 50 mM NaCl, and finally 2 volumes of 0.2 M NaCl both in the same buffer. During the final step, 10 μL (5 minute) fractions were collected. Fractions 73 to 118 inclusive were pooled, dialyzed against the 10 volumes of 10 mM sodium phosphate pH 6.0 twice and clarified by centrifugation at 100,000 g for 60 minutes.

II. Hydroxylapatite HPLC

Hydroxylapatite HPLC is not a technique hitherto used in isolating glial growth factors, but proved particularly efficacious in this invention.

The material obtained from the above CM-cellulose chromatography was filtered through a 0.22 μm filter (Nalgene), loaded at room temperature on to a high performance hydroxylapatite column (50 x 50 mm, Biorad) equipped with a guard column (15 x 25 mm, Biorad) and equilibrated with 10 mM potassium phosphate pH 6.0. Elution at room temperature was carried out at a flow rate of 2 $\mu l.minute^{-1}$ using the following programmed linear gradient:

time (min)	%B Solvent A : 10 mM potassium phosphate pH 6.0
0.0	0 Solvent B : 1.0 M potassium phosphate pH 6.0
5.0	0
7.0	20
70.0	20
150.0	100
180.0	100
185.0	0

6.0 μL (3 minutes) fractions were collected during the gradient elution. Fractions 39-45 were pooled and dialyzed against 10 volumes of 50 mM sodium phosphate pH 6.0.

III. Mono S FPLC

Mono S FPLC enabled a more concentrated material to be prepared for subsequent gel filtration.

Any particulate material in the pooled material from the hydroxylapatite column was removed by a clarifying spin at 100,000 g for 60 minutes prior to loading on to a preparative HR10/10 Mono S cation exchange column (100 x 10 mm, Pharmacia) which was then re-equilibrated to 50 MM sodium phosphate pH 6.0 at room temperature with a flow rate of 1.0 $\mu L.minute^{-1}$. Under these conditions, bound protein was eluted using the following programmed linear gradient:

time (min)	%B	Solvent A :	50 mM potassium phosphate pH 6. 0
0.0	0	Solvent B :	1.2 M sodium chloride, 50 mm
70.0	30		sodium phosphate pH 6.0
240.0	100		
250.0	100		
260.0	0		

1 μL (1 minute) fractions were collected throughout this gradient program. Fractions 99 to 115 inclusive were pooled.

IV. Gel Filtration FPLC

This step commenced the separation of the two factors of the invention prior to final purification, producing enriched fractions.

For the purposes of this step, a preparative Superose 12 FPLC column (510 x 20 mm, Pharmacia) was packed according to the manufacturers' instructions. In order to standardize this column, a theoretical plates measurement was made according to the manufacturers' instructions, giving a value of 9,700 theoretical plates.

The pool of Mono S eluted material was applied at room temperature in 2.5 μL aliquots to this column in 50 MM sodium phosphate, 0.75 NaCl pH 6.0 (previously passed through a C18 reversed phase column (Sep-pak, Millipore) at a flow rate of 1.0 $\mu L.minute^{-1}$. 1 μL (0.5 minute) fractions were collected from 35 minutes after each sample was applied to the column. Fractions 27 to 41 (GGF-II) and 42 to 57 (GGF-I) inclusive from each run were pooled.

V. Reversed-Phase HPLC

The GGF-I and GGF-II pools from the above Superose

12 runs were each divided into three equal aliquots. Each aliquot was loaded on to a C8 reversed-phase column (Aquapore RP-300 7 μ C8 220 x 4.6 mm, Applied Biosystems) protected by a guard cartridge (RP-8, 15 x 3.2 mm, Applied Biosystems) and equilibrated to 4°C at 0.5 μL.minute. Protein was eluted under these conditions using the following programmed linear gradient:

time (min)	%B		
(TFA)			
0		Solvent A :	0.1% trifluoroacetic acid
60	66.6	Solvent B :	90% acetonitrile, 0.1% TFA
62.0	100		
72.0	100		
75.0	0		

200 μL (0.4 minute) fractions were collected in siliconized tubes (Multilube tubes, Bioquote) from 15.2 minutes after the Beginning of the programmed gradient.

VI. SDS-Polyacrylamide Gel Electrophoresis

In this step, protein molecular weight standards, low range, catalogue no. 161-0304, from Bio-Rad Laboratories Limited, Watford, England were employed. The actual proteins used, and their molecular weight standards, have been listed hereinbefore.

Fractions 47 to 53 (GGF-I) and fractions 61 to 67 (GGF-II) from the reversed-phase runs were individually pooled. 7 μL of the pooled material was boiled in an equal volume of 0.0125 M Tris-Cl, 4% SDS, 20% glycerol, and 10% ß-mercaptoethanol for GGF-I, for 5 minutes and loaded on to an 11% polyacrylamide Leammli gel with a 4% stacking gel and run at a constant voltage of 50 V for 16 hours. This gel was then fixed and stained using a silver staining kit (Amersham). Under these conditions, the factors are each

seen as a somewhat diffuse band at relative molecular weights 30,000 to 36,000 Daltons (GGF-I) and 55,000 to 63,000 Daltons (GGF-II), as defined by molecular weight markers. From the gel staining, it is apparent that there are a small number of other protein species present at equivalent levels to the GGF-I and GGF-II species in the material pooled from the reversed-phase runs.

VII. Stability in Trifluoroacetic Acid

Stability data were obtained for the present Factors in the presence of trifluoroacetic acid, as follows:

GGF-I

Material from the reversed-phase HPLC, in the presence of 0.1% TFA and acetonitrile, was assayed within 12 hours of the completion of the column run and then after 10 weeks incubation at 40°C. Following incubation, the GGF-I had at least 50% of the activity of that material assayed directly off the column.

GGF-II

Material from the reversed-phase HPLC, in the presence of 0.1% TFA and acetonitrile, and stored at -20°C, was assayed after thawing and then after 4 days incubation at 40°C. Following incubation, the GGF-II had at least 50% of the activity of that material freshly thawed.

It will be appreciated that the trifluoroacetic acid concentration used in the above studies is that most commonly used for reversed-phase chromatography.

EXAMPLE 2

Amino acid sequences purified GGF-I and GGF-II

Amino acid sequence analysis studies were performed using highly purified bovine pituitary GGF-I and GGF-II. The conventional single letter code was used to describe the sequences. Peptides were obtained by lysyl endopeptidase and protease V8 digests, carried out on

reduced and carboxymethylated samples, with the lysyl endopeptidase digest of GGF-II carried out on material eluted from the 55-65 RD region of a 11% SDS-PAGE (MW relative to the above-quoted markers).

A total of 21 peptide sequences (see Figure 9) were obtained for GGF-I, of which 12 peptides (see Figure 10) are not present in current protein databases and therefore represent unique-sequences. A total of 12 peptide sequences (see Figure 11) were obtained for GGF-II, of which 10 peptides (see Figure 12) are not present in current protein databases and therefore represent unique sequences (an exception is peptide GGF-II 06 (SEQ ID No. 38) which shows identical sequences in many proteins which are probably of no significance given the small number of residues). These novel sequences are extremely likely to correspond to portions of the true amino acid sequences of GGFs I and II.

Particular attention can be drawn to the sequences of GGF-I 07 (SEQ ID No. 39) and GGF-II 12 (SEQ ID No. 44), which are clearly highly related. The similarities indicate that the sequences of these peptides are almost certainly those of the assigned GGF species, and are most unlikely to be derived from contaminant proteins.

In addition, in peptide GGF-II 02 (SEQ ID No. 34), the sequence X S S is consistent with the presence of an N linked carbohydrate moiety on an asparagine at the position denoted by X.

In general, in Figures 9 and 11, X represents an unknown residue denoting a sequencing cycle where a single position could not be called with certainty either because there was more than one signal of equal size in the cycle or because no signal was present. As asterisk denotes those peptides where the last amino acid called corresponds to the last amino acid present in that peptide. In the remaining peptides, the signal strength after the last

amino acid called was insufficient to continue sequence calling to the end of that peptide. The right hand column indicates the results of a computer database search using the GCG package FASTA and TFASTA programs to analyze the NBRF and EMBL sequence databases. The name of a protein in this column denotes identity of a portion of its sequence with the peptide amino acid sequence called allowing a maximum of two mismatches. A question mark denotes three mismatches allowed. The abbreviations used are as follows:

HMG-1	High Mobility Group protein-1
HMG-2	High Mobility Group protein-2
LH-alpha	Luteinizing hormone alpha subunit
LH-beta	Luteinizing hormone beta subunit

EXAMPLE 3

Mitogenic activity of purified GGF-I and GGF-II

The mitogenic activity of a highly purified sample containing both GGFs I and II was studied using a quantative method, which allows a single microculture to be examined for DNA synthesis, cell morphology, cell number and expression of cell antigens. This technique has been modified from a method previously reported by Muir et al. ((1990) Analytical Biochemistry 185:377-382). The main modifications are: 1) the use of uncoated microtiter plates, 2) the cell number per well, 3) the use of 5% Fetal Bovine Plasma (FBP) instead of 10% Fetal Calf Serum (FCS), and 4) the time of incubation in presence of mitogens and bromodeoxyuridine (BrdU), added simultaneously to the cultures. In addition the cell monolayer was not washed before fixation to avoid loss of cells, and the incubation time of monoclonal mouse anti-BrdU antibody and peroxidase conjugated goat anti-mouse immunoglobulin (IgG) antibody were doubled to increase the sensitivity of the assay. The assay, optimized for rat sciatic nerve Schwann cells, has also been used for several cell lines, after appropriate

modifications to the cell culture conditions.

I. Methods of Mitogenesis Testing

On day 1, purified Schwann cells were plated onto uncoated 96 well plates in 5% FBP/Dulbecco's Modified Eagle Medium (DMEM) (5,000 cells/well). On day 2, GGFs or other test factors were added to the cultures, as well as BrdU at a final concentration of 10mm. After 48 hours (day 4) BrdU incorporation was terminated by aspirating the medium and cells were fixed with 200 µl/well of 70% ethanol for 20 min at room temperature. Next, the cells were washed with water and the DNA denaturated by incubation with 100 µl 2N HCl for 10 min at 37°C. Following aspiration, residual acid was neutralized by filling the wells with 0.1M borate buffer, pH 9.0, and the cells were washed with phosphate buffered saline (PBS). Cells were then treated with 50 µl of blocking buffer (PBS containing 0.1% Triton X 100 and 2% normal goat serum) for 15 min at 37°C. After aspiration, monoclonal mouse anti-BrdU antibody (Dako Corp., Santa Barbara, CA) (50 µl/well, 1.4 mg/ml diluted in blocking buffer) was added and incubated for two hours at 37°C. Unbound antibodies were removed by three washes in PBS containing 0.1% Triton X-100 and peroxidase-conjugated goat ant-mouse IgG antibody (Dako Corp., Santa Barbara, CA) (50 µl/well, 2 mg/ml diluted in blocking buffer) was added and incubated for one hour at 37°C. After three washes in PBS/Triton and a final rinse in PBS, wells received 100 µl/well of 50 mM phosphate/citrate buffer, pH 5.0, containing 0.05% of the soluble chromogen o-phenylenediamine (OPD) and 0.02% H_2O_2. The reaction was terminated after 5-20 min at room temperature, by pipetting 80 µl from each well to a clean plate containing 40 µl/well of 2N sulfuric acid. The absorbance was recorded at 490nm using a plate reader (Dynatech Labs). The assay plates containing the cell monolayers were washed twice with PBS

and immunocytochemically stained for BrdU-DNA by adding 100 µl/well of the substrate diaminobenzidine (DAB) and 0.02% H_2O_2 to generate an insoluble product. After 10-20 min the staining reaction was stopped by washing with water, and BrdU-positive nuclei observed and counted using an inverted microscope. Occasionally, negative nuclei were counterstained with 0-001% Toluidine blue and counted as before.

II. Cell Lines used for Mitogenesis Assays

Swiss 3T3 Fibroblasts

Cells, from Flow Labs, were maintained in DMEM supplemented with 10% FCS, penicillin and streptomycin, at 37°C in a humidified atmosphere of 10% CO_2 in air. Cells were fed or subcultured every two days. For mitogenic assay, cells were plated at a density of 5,000 cells/well in complete medium and incubated for a week until cells were confluent and quiescent. The serum containing medium was removed and the cell monolayer washed twice with serum free-medium. 100 µl of serum free medium containing mitogens and 10µM BrdU were added to each well and incubated for 48 hours. Dose responses to GGFs and serum or PDGF (as a positive control) were performed.

BHK (Baby Hamster Kidney) 21 C13 Fibroblasts

Cells from European Collection of Animal Cell Cultures (ECACC), were maintained in Glasgow Modified Eagle Medium (GMEM) supplemented with 5% tryptose phosphate broth, 5% FCS, penicillin and streptomycin, at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were fed or subcultured every two to three days. For mitogenic assay, cells were plated at a density of 2,000 cell/well in complete medium for 24 hours. The serum containing medium was then removed and after washing with serum free medium, replaced with 100 µl of 0.1% FCS containing GMEM or GMEM

alone. GGFs and FCS or BFGF as positive controls were added, coincident with 10μM BrdU, and incubated for 48 hours. Cell cultures were then processed as described for Schwann cells.

C6 Rat Glioma Cell Line

Cells, obtained at passage 39, were maintained in DMEM containing 5% FCS, 5% Horse serum (HS), penicillin and streptomycin, at 37°C in a humidified atmosphere of 10% CO_2 in air. Cells were fed or subcultured every three days. For mitogenic assay, cells were plated at a density of 2,000 cells/well in complete medium and incubated for 24 hours. Then medium was replaced with a mixture of 1:1 DMEM and F12 medium containing 0.1% FCS, after washing in serum free medium. Dose responses to GGFs, FCS and AFGF were then performed and cells were processed through the ELISA as previously described for the other cell types.

PC12 (Rat Adrenal Pheochromocytoma Cells)

Cells from ECACC, were maintained in RPMI 1640 supplemented with 10% HS, 5% FCS, penicillin and streptomycin, in collagen coated flasks, at 370C in a humidified atmosphere of 5% CO_2 in air. Cells were fed every three days by replacing 80% of the medium. For mitogenic assay, cells were plated at a density of 3,000 cells/well in complete medium, on collagen coated plates (50 μl/well collagen, Vitrogen Collagen Corp., diluted 1 : 50, 30 min at 37°C) and incubated for 24 hours. The medium was then placed with fresh RPMI either alone or containing 1 mM insulin or 1% FCS. Dose responses to FCS/HS (1:2) as positive control and to GGFs were performed as before. After 48 hours cells were fixed and the ELISA performed as previously described.

III. Results of Mitogenesis Assays

All the experiments presented in this Example were performed using a highly purified sample from a Superose 12 chromatography purification step (see Example 1, section D) containing a mixture of GGF-I and GGF-II (GGFs).

Firstly, the results obtained with the BrdU incorporation assay were compared with the classical mitogenic assay for Schwann cells based on ^{125}I-UdR incorporation into DNA of dividing cells, described by J.P.Brockes ((1987) Methods Enzymol. 147:217).

Figure 13 shows the comparison of data obtained with the two assays, performed in the same cell culture conditions (5,000 cells/well, in 5% FBP/DMEM, incubated in presence of GGFs for 48hrs). As clearly shown, the results are comparable, but BrdU incorporation assay appears to be slightly more sensitive, as suggested by the shift of the curve to the left of the graph, i.e. to lower concentrations of GGFS.

As described under the section "Methods", after the immunoreactive BrdU-DNA has been quantitated by reading the intensity of the soluble product of the OPD peroxidase reaction, the original assay plates containing cell monolayers can undergo the second reaction resulting in the insoluble DAB product, which stains the BrdU positive nuclei. The microcultures can then be examined under an inverted microscope, and cell morphology and the numbers of BrdU-positive and negative nuclei can be observed.

In Figure 14a and Figure 14b the BrdU-DNA immunoreactivity, evaluated by reading absorbance at 490 nm, is compared to the number of BrdU-positive nuclei and to the percentage of BrdU-positive nuclei on the total number of cells per well, counted in the same cultures. Standard deviations were less than 10%. The two evaluation methods show a very good correlation and the discrepancy between the values at the highest dose of GGFs can be

explained by the different extent of DNA synthesis in cells detected as BrdU-positive.

The BrdU incorporation assay can therefore provide additional useful information about the biological activity of GGFs on Schwann cells when compared to the ^{125}I-UdR incorporation assay. For example, the data reported in Figure 15 show that GGFs can act on Schwann cells to induce DNA synthesis, but at lower doses to increase the number of negative cells present in the microculture after 48 hours.

The BrdU incorporation assay has been used on several cell lines of different origin. In Figure 16 the mitogenic responses of Schwann cells and Swiss 3T3 fibroblasts to GGFs are compared; despite the weak response obtained in 3T3 fibroblasts, some clearly BrdU-positive nuclei were detected in these cultures. Control cultures were run in parallel in presence of several doses of FCS or human recombinant PDGF, showing that the cells could respond to appropriate stimuli (not shown).

The ability of fibroblasts to respond to GGFs was further investigated using the BHK 21 C13 cell line. These fibroblasts, derived from kidney, do not exhibit contact inhibition or reach a quiescent state when confluent. Therefore the experimental conditions were designed to have a very low background proliferation without comprising the cell viability. GGFs have a significant mitogenic activity on BHK21 C13 cells as shown by Figure 17 and Figure 18. Figure 17 shows the Brdu incorporation into DNA by BHK 21 C13 cells stimulated by GGFS in the presence of 0.1% FCS. The good mitogenic response to FCS indicates that cell culture conditions were not limiting. In Figure 18 the mitogenic effect of GGFs is expressed as the number of BrdU-positive and BrdU-negative cells and as the total number of cells counted per well. Data are representative of two experiments run in duplicates; at least three fields per well were counted. As observed for Schwann cells in

addition to a proliferative effect at low doses, GGFs also increase the numbers of nonresponding cells surviving. The percentage of BrdU positive cells is proportional to the increasing amounts of GGFs added to the cultures. The total number of cells after 48 hours in presence of higher doses of GGFs is at least doubled, confirming that GGFs induce DNA synthesis and proliferation in BHK21 C13 cells. Under the same conditions, cells maintained for 48 hours in the presence of 2% FCS showed an increase of about six fold (not shown).

C6 glioma cells have provided a useful model to study glial cell properties. The phenotype expressed seems to be dependent on the cell passage, the cells more closely resembling an astrocyte phenotype at an early stage, and an oligodendrocyte phenotype at later stages (beyond passage 70). C6 cells used in these experiments were from passage 39 to passage 52. C6 cells are a highly proliferating population, therefore the experimental conditions were optimized to have a very low background of BrdU incorporation. The presence of 0.1% serum was necessary to maintain cell viability without significantly affecting the mitogenic responses, as shown by the dose response to FCS (Figure 19).

In Figure 20 the mitogenic responses to aFGF (acidic Fibroblast growth factor) and GGFs are expressed as the percentages of maximal BrdU incorporation obtained in the presence of FCS (8%). Values are averages of two experiments, run in duplicates. The effect of GGFs was comparable to that of a pure preparation of aFGF. aFGF has been described as a specific growth factor for C6 cells (Lim R. et al. (1990) Cell Regulation 1:741-746) and for that reason it was used as a positive control. The direct counting of BrdU positive and negative cells was not possible because of the high cell density in the microcultures. In contrast to the cell lines so far

reported, PC12 cells did not show any evident responsiveness to GGFS, when treated under culture conditions in which PC12 could respond to sera (mixture of FCS and HS as used routinely for cell maintenance). Nevertheless the number of cells plated per well seems to affect the behavior of PC12 cells, and therefore further experiments are required.

EXAMPLE 4

Isolation and cloning of nucleotide sequences encoding proteins containing GGF-I and GGF-II peptides

Isolation and cloning of the GGF-II nucleotide sequences was performed as outlined herein, using peptide sequence information and library screening, and was performed as set out below. It will be appreciated that the peptides of Figures 4 and 5 can be used as the starting point for isolation and cloning of GGF-I sequences by following the techniques described herein. Indeed, Figure 21, (SEQ ID No. 54-88) shows possible degenerate oligonucleotide probes for this purpose, and Figure 23, (SEQ ID Nos. 90-119), lists possible PCR primers. DNA sequence and polypeptide sequence should be obtainable by this means as with GGF-II, and also DNA constructs and expression vectors incorporating such DNA sequence, host cells genetically altered by incorporating such constructs/vectors, and protein obtainable by cultivating such host cells. The invention envisages such subject matter.

I. Design and Synthesis of oligonucleotide Probes and Primers

Degenerate DNA oligomer probes were designed by backtranslating the amino acid sequences (derived from the peptides generated from purified GGF protein) into nucleotide sequences. Oligomers represented either the coding strand or the non-coding strand of the DNA sequence.

When serine, arginine or leucine were included in the oligomer design, then two separate syntheses were prepared to avoid ambiguities. For example, serine was encoded by either TCN or AGY as in 537 and 538 or 609 and 610. Similar codon splitting was done for arginine or leucine (e.g. 544, 545). DNA oligomers were synthesized on a Biosearch 8750 4-column DNA synthesizer using ß cyanoethyl chemistry operated at 0.2 micromole scale synthesis. Oligomers were cleaved off the column (500 angstrom CpG resins) and deprotected in concentrated ammonium hydroxide for 6-24 hours at 55-60'C. Deprotected oligomers were dried under vacuum (Speedvac) and purified by electrophoresis in gels of 15% acrylamide (20 mono : 1 bis), 50 mM Tris-borate-EDTA buffer containing 7M urea. Full length oligomers were detected in the gels by UV shadowing, then the bands were excised and DNA oligomers eluted into 1.5 µls H20 for 4-16 hours with shaking. The eluate was dried, redissolved in 0.1 µl H_2O and absorbance measurements were taken at 260nm.

Concentrations were determined according to the following formula:

(A 260 x units/µl) (60.6/length) = x µM

All oligomers were adjusted to 50 µM concentration by addition of H_2O.

Degenerate probes designed as above are shown in Figure 21, (SEQ ID Nos. 54-88).

PCR primers were prepared by essentially the same procedures that were used for probes with the following modifications. Linkers of thirteen nucleotides containing restriction sites were included at the 5' ends of the degenerate oligomers for use in cloning into vectors. DNA synthesis was performed at 1 micromole scale using 1,000 angstrom CpG resins and inosine was used at positions where all four nucleotides were incorporated normally into degenerate probes. Purifications of PCR primers included

an ethanol precipitation following the gel electrophoresis purification.

II. Library Construction and Screening

A bovine genomic DNA library was purchased from Stratagene (Catalogue Number: 945701). The library contained 2 x 10^6 15-20kb Sau3Al partial bovine DNA fragments cloned into the vector lambda DashII. A bovine total brain CDNA library was purchased from Clonetech (Catalogue Number: BL 10139). Complementary DNA libraries were constructed (In Vitrogen; Stratagene) from mRNA prepared from bovine total brain, from bovine pituitary and from bovine posterior pituitary. In Vitrogen prepared two cDNA libraries: one library was in the vector lambda g10, the other in vector pcDNAI (a plasmid library). The Stratagene libraries were prepared in the vector lambda unizap. Collectively, the cDNA libraries contained 14 million primary recombinant phage.

The bovine genomic library was plated on E. coli K12 host strain LE392 on 23 x 23 cm plates (Nunc) at 150,000 to 200,000 phage plaques per plate. Each plate represented approximately one bovine genome equivalent. Following an overnight incubation at 37°c, the plates were chilled and replicate filters were prepared according to procedures of Grunstein and Hogness ((1975) PNAS (USA) 72:3961). Four plaque lifts were prepared from each plate onto uncharged nylon membranes (Pall Biodyne A or MSI Nitropure). The DNA was immobilized onto the membranes by cross-linking under UV light for 5 minutes or, by baking at 80°C under vacuum for two hours. DNA probes were labelled using T4 polynucleotide kinase (New England Biolabs) with gamma ^{32}P ATP (New England Nuclear; 6500 Ci/mmol) according to the specifications of the suppliers. Briefly, 50 pmols of degenerate DNA oligomer were incubated in the presence of 600 μCi gamma ^{32}P-ATP and 5 units T4 polynucleotide kinase

for 30 minutes at 37°C. Reactions were terminated, gel electrophoresis loading buffer was added and then radiolabelled probes were purified by electrophoresis. ^{32}P labelled probes were excised from gel slices and eluted into water. Alternatively, DNA probes were labelled via PCR amplification by incorporation of $\alpha^{32}P$-dATP or $\alpha^{32}P$ dCTP according to the protocol of Schowalter and Sommer ((1989) Anal. Biochem 177:90-94). Probes labelled in PCR reactions were purified by desalting on Sephadex G-150 columns.

Prehybridization and hybridization were performed in GMC buffer (0.52M NaPi, 7% SDS, 1% BSA, 1.5mM EDTA, O.1MNaCl 10μg/μl TRNA). Washing was performed in buffer A oligowash (160μl 1M $Na2HPO_4$, 200 μl 20% SDS, 8.0 μl 0.5m EDTA, 100 μl 5M NaCl, 3632 μl H20). Typically, 20 filters (400 sq. centimetres each) representing replicate copies of ten bovine genome equivalents were incubated in 200 μl hybridization solution with 100 pmols of degenerate oligonucleotide probe (128-512 fold degenerate). Hybridization was allowed to occur overnight at 5°C below the minimum melting temperature calculated for the degenerate probe. The calculation of minimum melting temperature assumes 2°C for an AT pair and 4°C for a GC pair.

Filters were washed in repeated changes of oligowash at the hybridization temperatures for four to five hours and finally, in 3.2M tetramethylammonium chloride, 1% SDS twice for 30 min at a temperature dependent on the DNA probe length. For 20mers, the final wash temperature was 60°C. Filters were mounted, then exposed to X-ray film (Kodak XAR5) using intensifying screens (Dupont Cronex Lightening Plus). Usually, a three to five day film exposure at minus 80°C was sufficient to detect duplicate signals in these library screens. Following analysis of the results, filters could be stripped and reprobed. Filters were stripped by incubating through two successive

cycles of fifteen minutes in a microwave oven at full power in a solution of 1% SDS containing 10mM EDTA pH8. Filters were taken through at least three to four cycles of stripping and reprobing with various probes.

III. Recombinant Phage Isolation, Growth and DNA Preparation

These procedures followed standard protocol as described in Recombinant DNA (Maniatis et al. Recombinant DNA 2:60-62:81).

IV. Analysis of Isolated Clones Using DNA Digestion and Southern Blots

Recombinant Phage DNA samples (2 micrograms) were digested according to conditions recommended by the restriction endonuclease supplier (New England Biolabs). Following a four hour incubation at 37°C, the reactions products were precipitated in the presence of 0.1M sodium acetate and three volumes of ethanol. Precipitated DNA was collected by centrifugation, rinsed in 75% ethanol and dried. All resuspended samples were loaded onto agarose gels (typically 1% in TAE buffer; 0.04M Tris acetate, 0.002M EDTA). Gel runs were at 1 volt per centimetre from 4 to 20 hours. Markers included lambda Hind III DNA fragments and/or ØX174HaeIII DNA fragments (New England Biolabs). The gels were stained with 0.5 micrograms/μl of ethidium bromide and photographed. For southern blotting, DNA was first depurinated in the gel by treatment with 0.125 N HCl, denatured in 0.5 N NaOH and transferred in 20x SSC (3M sodium chloride, 0.03 M sodium citrate) to uncharged nylon membranes. Blotting was done for 6 hours up to 24 hours, then the filters were neutralized in 0.5M Tris HCl pH 7.5, 0.15 M sodium chloride, then rinsed briefly in 50 mM Tris-borate EDTA.

For cross-linking, the filters were wrapped first in transparent plastic wrap, then the DNA side exposed for

five minutes to an ultraviolet light. Hybridization and washing was performed as described for library screening (see section 2 of this Example). For hybridization analysis to determine whether similar genes exist in other species slight modifications were made. The DNA filter was purchased from Clonetech (Catalogue Number 7753-1) and contains 5 micrograms of EcoRI digested DNA from various species per lane. The probe was labelled by PCR amplification reactions as described in section 2 above, and hybridizations were done in 80% buffer B(2g polyvinylpyrrolidine, 2g Ficoll-400, 2g bovine serum albumin, 50μl 1M Tris-HCl(pH 7.5) 58g NaCl, 1g sodium pyrophosphate, log sodium dodecyl sulfate, 950μl H_2O) containing 10% dextran sulfate. The probes were denatured by boiling for ten minutes then rapidly cooling in ice water. The probe was added to the hybridization buffer at 10^6 dpm ^{32}P per μl and incubated overnight at 60°C. The filters were washed at 60°C first in buffer B followed by 2X SSC, 0.1% SDS then in 1x SSC, 0.1% SDS. For high stringency, experiments, final washes were done in 0.1 x SSC, 1% SDS and the temperature raised to 65°C.

Southern blot data were used to prepare a restriction map of the genomic clone and to indicate which subfragments hybridized to the GGF probes (candidates for subcloning).

V. Subcloning of Pieces of DNA Homologous to Hybridization Probes

DNA digests (e.g. 5 micrograms) were loaded onto 1% agarose gels then appropriate fragments excised from the gels following staining. The DNA was purified by adsorption onto glass beads followed by elution using the protocol described by the supplier (Bio 101). Recovered DNA fragments -(100-200 ng) were ligated into linearized dephosphorylated vectors, e.g. pT3T7 (Ambion), which is a derivative of pUC18, using T4 ligase (New England Biolabs).

This vector carries the E. coli ß lactamase gene, hence, transformants can be selected on plates containing ampicillin. The vector also supplies ß-galactosidase complementation to the host cell, therefore non-recombinants (blue) can be detected using isopropylthiogaloctoside and Bluogal (Bethesda Research Labs). A portion of the ligation reactions was used to transform E. coli K12 XL1 blue competent cells (Stratagene Catalogue Number: 200236) and then the transformants were selected on LB plates containing 50 micrograms per µl ampicillin. White colonies were selected and plasmid mini preps were prepared for DNA digestion and for DNA sequence analysis. Selected clones were retested to determine if their insert DNA hybridized with the GGF probes.

VI. DNA Sequencing

Double stranded plasmid DNA templates were prepared from double stranded plasmids isolated from 5µl cultures according to standard protocols. Sequencing was by the dideoxy chain termination method using Sequenase 2.0 and a dideoxynucleotide sequencing kit (US Biochemical) according to the manufacturers protocol (a modification of Sanger et al. (1977) PNAS (USA) 74:5463). Alternatively, sequencing was done in a DNA thermal cycler (Perkin Elmer, model 4800) using a cycle sequencing kit (New England Biolabs; Bethesda Research Laboratories) and was performed according to manufacturers instructions using a 5'-end labelled primer. Sequence primers were either those supplied with the sequencing kits or were synthesized according to sequence determined from the clones. Sequencing reactions were loaded on and resolved on 0.4mm thick sequencing gels of 6% polyacylamide. Gels were dried and exposed to X-Ray film. Typically, 35S was incorporated when standard sequencing kits were used and a ^{32}P end labelled primer was used for cycle sequencing reactions. Sequences were read into a DNA

sequence editor from the bottom of the gel to the top (5′ direction to 3′) and data were analyzed using programs supplied by Genetics Computer Group (GCG, University of Wisconsin).

VII. RNA Preparation and PCR Amplification

Open reading frames detected in the genomic DNA and which contained sequence encoding GGF peptides were extended via PCR amplification of pituitary RNA. RNA was prepared from frozen bovine tissue (Pelfreeze) according to the guanidine neutral-CsCl chloride procedure (Chirgwin et. al. (1979) Biochemistry 18:5294). Polyadenylated RNA was selected by oligo-dT cellulose column chromatography (Aviv and Leder. (1972) PNAS (USA) 69:1408).

Specific target nucleotide sequences were amplified beginning with either total RNA or polyadenylated RNA samples that had been converted to cDNA using the Perkin Elmer PCR/RNA Kit Number: N808-0017. First strand reverse transcription reactions used 1 μg template RNA and either primers of oligo dT with restriction enzyme recognition site linkers attached or specific antisense primers determined from cloned sequences with restriction sites attached. To produce the second strand, the primers either were plus strand unique sequences as used in 3′ RACE reactions (Frohman et. al. (1988) PNAS (USA) 85:8998) or were oligo dT primers with restriction sites attached if the second target site had been added by terminal transferase tailing first strand reaction products with dATP (e.g. 5′ race reactions, Frohman et. al., ibid). Alternatively, as in anchored PCR reactions the second strand primers were degenerate, hence, representing particular peptide sequences.

The amplification profiles followed the following general scheme: 1) five minutes soak file at 95°C; 2) thermal cycle file of 1 minute, 95°C; 1 minute ramped down to an annealing temperature of 45°C, 50°C or 55°C; maintain

the annealing temperature for one minute; ramp up to 72°C over one minute; extend at 72°C for one minute or for one minute plus a 10 second auto extension; 3) extension cycle at 72°C, five minutes, and; 4) soak file 40°C for infinite time. Thermal cycle files (#2) usually were run for 30 cycles. Sixteen μl of each 100 μl amplification reaction was analyzed by electrophoresis in 2% Nusieve 1% agarose gels run in TAE buffer at 4 volts per centimetre for three hours. The gels were stained, then blotted to uncharged nylon membranes which were probed with labelled DNA probes that were internal to the primers.

Specific sets of DNA amplification products could be identified in the blotting experiments and their positions used as a guide to purification and reamplification. When appropriate, the remaining portions of selected samples were loaded onto preparative gels, then following electrophoresis four to five slices of 0.5 mm thickness (bracketing the expected position of the specific product) were taken from the gel. The agarose was crushed, then soaked in 0.5 μl of electrophoresis buffer from 2-16 hours at 40°C. The crushed agarose was centrifuged for two minutes and the supernate was transferred to fresh tubes.

Reamplification was done on five microlitres (roughly 1% of the product) of the eluted material using the same sets of primers and the reaction profiles as in the original reactions. When the reamplification reactions were completed, samples were extracted with chloroform and transferred to fresh tubes. Concentrated restriction enzyme buffers and enzymes were added to the reactions in order to cleave at the restriction sites present in the linkers. The digested PCR products were purified by gel electrophoresis, then subcloned into vectors as described in the subcloning section above. DNA sequencing was done described as above.

VII. DNA Sequence Analysis

DNA sequences were assembled using a fragment assembly program and the amino acid sequences deduced by the GCG programs GelAssemble, Map and Translate. The deduced protein sequences were used as a query sequence to search protein sequence databases using WordSearch. Analysis was done on a VAX Station 3100 workstation operating under VMS 5.1. The database search was done on SwissProt release number 21 using GCG Version 7.0.

VII. Results

As indicated, to identify the DNA sequence encoding bovine GGF-II degenerate oligonucleotide probes were designed from GGF-II peptide sequences. GGF-II 12 (SEQ ID No. 44), a peptide generated via lysyl endopeptidase digestion of a purified GGF-II preparation (see Figures 11 and 12) showed strong amino acid sequence homology with GGF-I 07 (SEQ ID No. 39), a tryptic peptide generated from a purified GGF-I preparation. GGF-II 12 was thus used to create ten degenerate oligonucleotide probes (see oligos 609, 610 and 649 to 656 in Figure 21, SEQ ID Nos. 69-71 and 79, respectively). A duplicate set of filters were probed with two sets (set 1=609, 610; set 2=649-656) of probes encoding two overlapping portions of GGF-II 12. Hybridization signals were observed, however, only one clone hybridized to both probe sets. The clone (designated GGF2BG1) was purified.

Southern blot analysis of DNA from the phage clone GGF2BG1 confirmed that both sets of probes hybridized with that bovine DNA sequence, and showed further that both probes reacted with the same set of DNA fragments within the clone. Based on those experiments a 4 kb Eco RI sub-fragment of the original clone was identified, subcloned and partially sequenced. Figure 22 shows the nucleotide sequence and the deduced amino acid sequence

(SEQ ID No. 89) of the initial DNA sequence readings that included the hybridization sites of probes 609 and 650, and confirmed that a portion of this bovine genomic DNA encoded peptide 12 (KASLADSGEYM).

Further sequence analysis demonstrated that GGF-II 12 resided on a 66 amino acid open reading frame (see below) which has become the starting point for the isolation of overlapping sequences representing a putative bovine GGF-II gene and a cDNA.

Several PCR procedures were used to obtain additional coding sequences for the putative bovine GGF-II gene. Total RNA and oligo dT-selected (poly A containing) RNA samples were prepared from bovine total pituitary, anterior pituitary, posterior pituitary, and hypothalamus. Using primers from the list shown in Figure 23 (SEQ ID No. 109-119) one-sided PCR reactions (RACE) were used to amplify cDNA ends in both the 3′ and 5′ directions, and anchored PCR reactions were performed with degenerate oligonucleotide primers representing additional GGF-II peptides. Figure 24 summarizes the contiguous DNA structures and sequences obtained in those experiments. From the 3′ RACE reactions, three alternatively spliced CDNA sequences were produced, which have been cloned and sequenced. A 5′ RACE reaction led to the discovery of an additional exon containing coding sequence for at least 52 amino acids. Analysis of that deduced amino acid sequence revealed peptides GGF-II-6 and a sequence similar to GGF-I-18 (see below). The anchored PCR reactions led to the identification of (cDNA) coding sequences of peptides GGF-II-1, 2, 3 and 10 contained within an additional cDNA segment of 300 bp. The 5′ limit of this segment (i.e. segment E, see Fig. 31) is defined by the oligonucleotide which encodes peptide GGF-II-1 and which is used in the PCR reaction. (Additional 5′ sequence data exists as described for the human clone in Example 6.) Thus this clone

contains nucleotide sequences encoding six out of the existing total of nine novel GGF-II peptide sequences.

The cloned gene was characterized first by constructing a physical map of GGF2BG1 that allowed positioning the coding sequences as they were found (see below, Figure 25). DNA probes from the coding sequences described above have been used to identify further DNA fragments containing the exons on this phage clone and to identify clones that overlap in both directions. The putative bovine GGF-II gene is divided into at least 5 coding segments, but only coding segments A and B have been defined as exons and sequenced and mapped thus far. The summary of the contiguous coding sequences identified is shown in Figure 26. The exons are listed (alphabetically) in the order of their discovery. It is apparent from the intron/exon boundaries that exon B may be included in cDNAs that connect coding segment E and coding segment A. That is, exon B cannot be spliced out without compromising the reading frame. Therefore, we suggest that three alternative splicing patterns can produce putative bovine GGF-II cDNA sequences 1, 2 and 3. The coding sequences of these, designated GGF2BPP1.CDS, GGF2BPP2.CDS and GGF2BPP3.CDS, respectively, are given in Figures 28a (SEQ ID No. 133), 28b (SEQ ID No. 134) and 28c (SEQ ID No. 135), respectively. The deduced amino acid sequence of the three cDNAs is also given in Figures 28a, 28b and 28c (SEQ ID Nos. 133-135, respectively).

The three deduced structures encode proteins of lengths 206, 281 and 257 amino acids. The first 183 residues of the deduced protein sequence are identical in all three gene products. At position 184 the clones differ significantly. A codon for glycine GGT in GGF2BPP1 also serves as a splice donor for GGF2BPP2 and GGF2BPP3, which alternatively add on exons C, C/D, C/D′ and D or C, C/D and D, respectively shown in Figure 33 (SEQ ID No. 149).

GGF2BPP1 is a truncated gene product which is generated by reading past the coding segment a splice junction into the following intervening sequence (intron). This represents coding segment A′ in Figure 31 (SEQ ID Nos. 140, 168). The transcript ends adjacent to a canonical AATAAA polyadenylation sequence, and we suggest that this truncated gene product represents a bona fide mature transcript. The other two longer gene products share the same 3′ untranslated sequence and polyadenylation site.

All three of these molecules contain six of the nine novel GGF-II peptide sequences (see Figure 12) and another peptide is highly homologous to GGF-I-18 (see Figure 27). This finding gives a high probability that this recombinant molecule encodes at least a portion of bovine GGF-II. Furthermore, the calculated isoelectric points for the three peptides are consistent with the physical properties of GGF-I and II. Since the molecular size of GGF-2 is roughly 60 kd, the longest of the three cDNAs should encode a protein with nearly one-half of the predicted number of amino acids.

A probe encompassing the B and A exons was labelled via PCR amplification and used to screen a cDNA library made from RNA isolated from bovine posterior pituitary. One clone (GGF2BPP5) showed the pattern indicated in Figure 30 and contained an additional DNA coding segment (G) between coding segments A and C. The entire nucleic acid sequence is shown in Figure 32 (SEQ ID No. 148). The predicted translation product from the longest open reading frame is 241 amino acids. A portion of a second cDNA (GGF2BPP4) was also isolated from the bovine posterior pituitary library using the probe described above. This clone showed the pattern indicated in Figure 30. This clone is incomplete at the 5′ end, but is a splicing variant in the sense that it lacks coding segments G and D. BPP4 also displays a novel 3′ end with regions H, K and L

beyond region C/D. The sequence of BPP4 is shown in Figure 34 (SEQ ID no. 150).

EXAMPLE 5

GGF Sequences in Various Species

Computer database searching has not revealed any meaningful similarities between any predicted GGF translation products and known protein sequences. This suggests that GGF-II is the first member of a new family or superfamily of proteins. In high stringency cross hybridization studies (DNA blotting experiments) with other mammalian DNAs we have shown clearly that DNA probes from this bovine recombinant molecule can readily detect specific sequences in a variety of samples tested. A highly homologous sequence is also detected in human genomic DNA. The autoradiogram is shown in Figure 29. The signals in the lanes containing rat and human DNA represent the rat and human equivalents of GGF, the sequences of which have been recently reported by Holmes et al. ((1992) Science 256:1205) and Wen et al. ((1992) Cell 69:559).

EXAMPLE 6

Isolation of a Human Sequence Encoding Human GGF2

Several human clones containing sequences homologous to the bovine GGFII coding segment E were isolated by screening a human cDNA library prepared from brain stem (Stratagene catalog #935206). This strategy was pursued based upon the strong link between most of the GGF2 peptides (unique to GGF2) and the predicted peptide sequence from clones containing the bovine E segment. This library was screened as described in Example 4, Section II using the oligonucleotide probes 914-919 listed below.

914 TCGGGCTCCATGAAGAAGATGTA

915 TCCATGAAGAAGATGTACCTGCT

916 ATGTACCTGCTGTCCTCCTTGA

917 TTGAAGAAGGACTCGCTGCTCA

918 AAAGCCGGGGGCTTGAAGAA

919 ATGARGTGTGGGCGGCGAAA

Clones detected with these probes were further analyzed by hybridization. A probe derived from coding segment A (see Figure 21), which was produced by labeling a polymerase chain reaction (PCR) product from segment A, was also used to screen the primary library. Several clones that hybridized with both A and E derived probes were selected and one particular clone, GGF2HBS5, was selected for further analysis. This clone is represented by the pattern of coding segments (EBACC/D'D as shown in Figure 31). The E segment in this clone is the human equivalent of the truncated bovine version of E shown in Figure 37. GGF2HBS5 is the most likely candidate to encode GGFII of all the "putative" GGFII candidates described. The length of coding sequence segment E is 786 nucleotides plus 264 bases of untranslated sequence. The predicted size of the protein encodied by GGF2HBS5 is approximately 423 amino acids (approximately 45 kilodaltons), which is similar to the size of the deglycosylated form of GGF (see Example 15). Additionally, seven of the GGFII peptides listed in Figure 27 have equivalent sequences which fall within the protein sequence predicted from region E. Peptides II-6 and II-12 are exceptions, which fall in coding segment B and coding segment A, respectively. RNA encoding the GGF2HBS5 protein was produced in an <u>in vitro</u> transcription system driven by the bacteriophage T7 promoter resident in the vector (Bluescript SK [Stratagene Inc.] see Figure 44) containing the GGF2HBS5 insert. This RNA can be translated in a cell free (rabbit reticulocyte) translation system and the size of the protein product was 45 Kd. Additionally, the cell-free product has been assayed in a Schwann cell mitogenic assay to confirm biological activity. Schwann cells treated with

conditioned medium show both increased proliferation as measured by incorporation of ^{125}-Uridine and phosphorylation on tyrosine of a protein in the 185 kilodalton range.

Thus the size of the product encoded by GGF2HBS5 and the presence of DNA sequences which encode human peptides highly homologous to the bovine peptides shown in Figure 12 confirm that GGF2HBS5 encodes the human equivalent of bovine GGF2. The fact that conditioned media prepared from cells transformed with this clone elicits Schwann cell mitogenic activity confirms that the GGFIIHBS5 gene product (unlike the BPP5 gene product) is secreted. Additionally the GGFBPP5 gene product seems to mediate the Schwann cell proliferation response via a receptor tyrosine kinase such as p185^{erbB2} or a closely related receptor (see Example 13).

EXAMPLE 7

Isolation of Human Sequences Related to Bovine GGF

The result in Example 5 indicates that GGF related sequences from human sources can also be easily isolated by using DNA probes derived from bovine GGF sequences. Alternatively, the procedure described by Holmes et al. ((1992) Science 256:1205) can be used. In this example a human protein (heregulin α) which binds to and activates the p185^{erbB2} receptor (and is related to GGF) is purified from a tumor cell line and the derived peptide sequence is used to produce oligonucleotide probes which were utilized to clone the cDNAs encoding heregulin. This is a similar approach to that used in examples 1-4 for the cloning of GGF sequences from pituitary cDNAs. The heregulin protein and complementary DNAs were isolated according to the following procedures. Heregulin was purified from medium conditioned by MDA-MB-231 breast cancer cells (ATCC #HTB 26) grown on Percell Biolytica microcarrier beads (Hyclone Labs). The medium (10 liters) was concentrated ~25-fold by filtration through a membrane (10-kD cutoff) (Millipore)

and clarified by centrifugation and filtration through a filter (0.22 μm). The filtrate was applied to a heparin Sepharose column (Pharmacia) and the proteins were eluted with steps of 0.3, 0.6, and 0.9 M NaCl in phosphate-buffered saline. Activity in the various chromatographic fractions was measured by quantifying the increase in tyrosine phosphorylation of $p185^{erbB2}$ in MCF-7 breast tumor cells (ATCC # HTB 22). MCF-7 cells were plated in 24-well Costar plates in F12 (50%) Dulbecco's minimum essential medium (50%) containing serum (10%) (10^5 cells per well), and allowed to attach for at least 24 hours. Prior to assay, cells were transferred into medium without serum for a minimum of 1 hour. Column fractions (10 to 100 μl) were incubated for 30 min. at 37°. Supernatants were then aspirated and the reaction was stopped by the addition of SDS-PAGE sample buffer 100 μl). Samples were heated for 5 min. at 100°C, and portions (10 to 15 μl) were applied to a tris-glycine gel (4 to 20%) (Novex). After electrophoresis, proteins were electroblotted onto a polyvinylidenedifluoride (PVDF) membrane and then blocked with bovine serum albumin (5%) in tris-buffered saline containing Tween-20 (0.05%) (TBST). Blots were probed with a monoclonal antibody (1:1000 dilution) to phosphotyrosine (Upstate Biotechnology) for a minimum of 1 hour at room temperature. Blots were washed with TBST, probed with an antibody to mouse immunoglobulin G conjugated to alkaline phosphatase (Promega) (diluted 1:7500) for a minimum of 30 min. at room temperature. Reactive bands were visualized with 5-bromo-4-chloro-3-indoyl-1-phosphate and nitro-blue tetrazolium. Immunoblots were scanned with a Scan Jet Plus (Hewlett-Packard) densitometer. Signal intensities for unstimulated MCF-7 cells were 20 to 30 units. Fully stimulated $p185^{erbB2}$ yielded signals of 180 to 200 units. The 0.6 M NaCl pool, which contained most of the activity,

was applied to a polyaspartic acid (PolyLC) column equilibrated in 17 mM sodium phosphate (pH 6.8) containing ethanol (30%). A linear gradient from 0.3 M to 0.6 M NaCl in the equilibration buffer was used to elute bound proteins. A peak of activity (at ~0.45 M NaCl) was further fractionated on a C4 reversed-phase column (SynChropak RP-4) equilibrated in buffer containing TFA (0.1%) and acetonitrile (15%). Proteins were eluted from this column with an acetonitrile gradient from 25 to 40% over 60 min. Fractions (1 μl) were collected, assayed for activity, and analyzed by SDS-PAGE on tris-glycine gels (4-20%, Novex).

HPLC-purified HRG-α was digested with lysine C in SDS (0.1%), 10 mM dithiothreitol, 0.1 M NH_4HCO_3 (pH 8.0) for 20 hours at 37°C and the resultant fragments were resolved on a Synchrom C4 column (4000Å, 0.2 by 10 cm). The column was equilibrated in 0.1% TFA and eluted with a 1-propanol gradient in 0.1% TFA (Henzel et al. (1989) J. Biol. Chem. 264:15905). Peaks from the chromatographic run were dried under vacuum and sequenced. One of the peptides (eluting at ~24% 1-propanol) gave the sequence [A]AEKEKTF[C]VNGGEXFMVKDLXNP (SEQ ID Nos 162). Residues in brackets were uncertain and an X represents a cycle in which it was not possible to identify the amino acid. The initial yield was 8.5 pmol and the sequence did not correspond to any known protein. Residues 1, 9, 15, and 22 were later identified in the cDNA sequence as cysteine. Direct sequencing of the ~45-kD band from a gel that had been overloaded and blotted onto a PVDF membrane revealed a low abundance sequence XEXKE[G][R]GK[G]K[G]KKKEXGXG[K] (SEQ ID No. 169) with a very low initial yield (0.2 pmol). This corresponded to amino acid residues 2 to 22 of heregulin-α (Fig. 31), suggesting that serine 2 is the NH_2-terminus of proHRG-α. Although the NH_2 terminus was blocked, it was observed that occasionally a small amount of a normally blocked protein may not be

post-translationally modified. The NH_2 terminal assignment was confirmed by mass spectrometry of the protein after digestion with cyanogen bromide. The COOH-terminus of the isolated protein has not been definitely identified; however, by mixture sequencing of proteolytic digests, the mature sequence does not appear to extend past residue 241. Abbreviations for amino residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T. Thr; V, Val; W, Trp; and Y, Tyr.

As a source of cDNA clones, an oligo(dT)-primed λgt10 (Hurn et al. (1984) λgt10 and λgt11 DNA Cloning Techniques: A Practical Approach) cDNA library was constructed (Gubler and Hoffman. (1983) Gene 25:263) with mRNA purified (Chirwin et al. (1979) Biochemistry 18:5294) from MDA-MB-231 cells. The following eightfold degenerate antisense deoxyoligonucleotide encoding the 13-amino acid sequence AEKEKTFCVNGGE (SEQ ID No. 164) was designed on the basis of human codon frequency optima (Lathe. (1985) J. Mol. Biol. 183:1) and chemically synthesized:

5′-CTCGCC (G OR T) CC (A OR G) TTCAC (A OR G) CAGAAGGTCTTCTCCTTCTCAGC-3′ (SEQ ID No. 165). For the purpose of probe design a cysteine was assigned to an unknown residue in the amino acid sequence. The probe was labeled by phosphorylation and hybridized under low-stringency conditions to the cDNA library. The proHRG-α protein was identified in this library. HRB-ß1 cDNA was identified by probing a second oligo(dT)-primed λgt10 library made from MDA-MB-231 cell mRNA with sequences derived from both the 5′ and 3′ ends of proHRG. Clone 13 IFig. 2A) was a product of screening a primed (5′-CCTCGCTCCTTCTTCTTGCCCTTC-3′ primer; proHRG-α antisense nucleotides 33 to 56) MDA-MB-231 λgt10 library with 5′ HRG sequence. A sequence corresponding to the 5′ end of clone 13 as the probe was used to identify proHRGß2 and proHRGß3

in a third oligo(dT)-primed λgt10 library derived from MDA-MB-231 cell mRNA. Two cDNA clones encoding each of the four HRGs were sequenced (Sanger et al. (1977) PNAS (USA) 74:5463). Another cDNA designated clone 84 has an amino acid sequence identical to proHRGß2 through amino acid 420. A stop codon at position 421 is followed by a different 3'-untranslated sequence.

EXAMPLE 8

Isolation of a Further Splicing Variant

The methods in Example 7 produced four closely related sequences (heregulin α, ß1, ß2, ß3) which arise as a result of splicing variation. Peles et al. ((1992) Cell 69:205) and Wen et al. ((1992) Cell 69:559) have isolated another splicing variant (from rat) using a similar purification and cloning approach to that described in Examples 1-4 and 7 involving a protein which binds to $p185^{erbB2}$. The cDNA clone was obtained as follows (via the purification and sequencing of a $p185^{erbB2}$ binding protein from a transformed rat fibroblast cell line).

A $p185^{erbB2}$ binding protein was purified from conditioned medium as follows. Pooled conditioned medium from three harvests of 500 roller bottles (120 liters total) was cleared by filtration through 0.2 μ filters and concentrated 31-fold with a Pelicon ultrafiltration system using membranes with a 20kd molecular size cutoff. All the purification steps were performed by using a Pharmacia fast protein liquid chromatography system. The concentrated material was directly loaded on a column of heparin-Sepharose (150 μl, preequilibrated with phosphate-buffered saline (PBS)). The column was washed with PBS containing 0.2 M NaCl until no absorbance at 280 nm wavelength could be detected. Bound proteins were then eluted with a continuous gradient (250 μl) of NaCl (from 0.2 M to 1.0 M), and 5 μl fractions were collected.

Samples (0.01 µl of the collected fractions) were used for the quantitative assay of the kinase stimulatory activity. Active fractions from three column runs (total volume = 360 µl) were pooled, concentrated to 25 µl by using a YM10 ultrafiltration membrane (Amicon, Danvers, MA), and ammonium sulfate was added to reach a concentration of 1.7 M. After clearance by centrifugation (10,000 x g, 15 min.), the pooled material was loaded on a phenyl-Superose column (HR10/10, Pharmacia). The column was developed with a 45 µl gradient of $(NH_4)_2SO_4$ (from 1.7 M to no salt) in 0.1 M Na_2PO_4 (pH 7.4), and 2 µl fractions were collected and assayed (0.002 µl per sample) for kinase stimulation (as described in Example 7). The major peak of activity was pooled and dialyzed against 50 mM sodium phosphate buffer (pH 7.3). A Mono-S cation-exchange column (HR5/5, Pharmacia) was preequilibrated with 50 mM sodium phosphate. After loading the active material (0.884 mg of protein; 35 µl), the column was washed with the starting buffer and then developed at a rate of 1 µl/min. with a gradient of NaCl. The kinase stimulatory activity was recovered at 0.45-0.55 M salt and was spread over four fractions of 2 µl each. These were pooled and loaded directly on a Cu^{+2} chelating columns (1.6 µl, HR2/5 chelating Superose, Pharmacia). Most of the proteins adsorbed to the resin, but they gradually eluted with a 30 µl linear gradient of ammonium chloride (0-1 M). The activity eluted in a single peak of protein at the range of 0.05 to 0.2 M NH_4Cl. Samples from various steps of purification were analyzed by gel electrophoresis followed by silver staining using a kit from ICN (Costa Mesa, CA), and their protein contents were determined with a Coomassie blue dye binding assay using a kit from Bio-Rad (Richmond, CA).

The p44 protein (10 µg) was reconstituted in 200 µl of 0.1 M ammonium bicarbonate buffer (pH 7.8). Digestion was conducted with L-1-tosyl-amide 2-phenylethyl

chloromethyl ketone-treated trypsin (Serva) at 37°C for 18 hr. at an enzyme-to-substrate ratio of 1:10. The resulting peptide mixture was separated by reverse phase HPLC and monitored at 215 nm using a Vydac C4 micro column (2.1 mm i.d. x 15 cm, 300) and an HP 1090 liquid chromatographic system equipped with a diode-array detector and a workstation. The column was equilibrated with 0.1% trifluoroacetic acid (mobile phase A), and elution was effected with a linear gradient from 0%-55% mobile phase B (90% acetonitrile in 0.1% trifluoroacetic acid) over 70 min. The flow rate was 0.2 μl/min. and the column temperature was controlled at 25°C. One-third aliquots of the peptide peaks collected manually from the HPLC system were characterized by N-terminal sequence analysis by Edman degradation. The fraction eluted after 27.7 min. (T27.7) contained mixed amino acid sequences and was further rechromatographed after reduction as follows: A 70% aliquot of the peptide fraction was dried in vacuo and reconstituted in 100 μl of 0.2 M ammonium bicarbonate buffer (pH 7.8). DTT (final concentration 2 mM) was added to the solution, which was then incubated at 37°C for 30 min. The reduced peptide mixture was then separated by reverse-phase HPLC using a Vydac column (2.1 mm i.d. x 15 cm). Elution conditions and flow rat were identical to those described above. Amino acid sequence analysis of the peptide was performed with a Model 477 protein sequencer (Applied Biosystems, Inc., Foster City, CA) equipped with an on-line phenylthiohydantoin (PTH) amino acid analyzer and a Model 900 data analysis system (Hunkapiller et al. (1986)). The protein was loaded onto a trifluoroacetic acid-treated glass fiber disc precycled with polybrene and NaCl. The PTH-amino acid analysis was performed with a micro liquid chromatography system (Model 120) using dual syringe pumps and reverse-phase (C-18) narrow bore columns (Applied Biosystems, 2.1 mm x 250 mm).

RNA was isolated from Rat1-EJ cells by standard procedures (Maniatis et al. (1982) Molecular Cloning: A Laboratory Manual) and poly $(A)^+$ was selected using an mRNA Separator kit (Clontech Lab, Inc., Palo, Alto, CA). cDNA was synthesized with the Superscript kit (from BRL Life Technologies, Inc., Bethesda, MS). Column-fractionated double-strand cDNA was ligated into an SalI- and NacI-digested pJT-2 plasmid vector, a derivative of the pCD-X vector (Okayama and Berg (1983) Mol. Cell Biol. 3:280) and transformed into DH10B E. coli cells by electroporation (Dower et al. (1988) Nucl. Acids Res. 16:6127). Approximately 5 x 10^5 primary transformants were screened with two oligonucleotide probes that were derived from the protein sequences of the N-terminus of NDF (residues 5-24) and the T40.4 tryptic peptide (residues 7-12). Their respective sequences were as follows (N indicates all 4 nt):

(1) 5'-ATA GGG AAG GGC GGG GGA AGG GTC NCC CTC NGC
A T
AGG GCC GGG CTT GCC TCT GGA GCC TCT-3'

(2) 5'-TTT ACA CAT ATA TTC NCC-3'
C G G C

(1: SEQ ID No. 167; 2: SEQ ID No. 168)

The synthetic oligonucleotides were end-labeled with [γ-^{32}P]ATP with T4 polynucleotide kinase and used to screen replicate sets of nitrocellulose filters. The hybridization solution contained 6 x SSC, 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 2 x Denhardt's solution, 50 μg/ml salmon sperm DNA, and 20% formamide (for probe 1) or no formamide (for probe 2). The filters were washed at either 50°C with 0.5 x SSC, 0.2% SDS, 2 mM EDTA (for probe 1) or at 37°C with 2 x SSC, 0.2% SDS, 2 mM EDTA (for probe 2). Autoradiography of the filters gave ten clones that hybridized with both probes.

These clones were purified by replating and probe hybridization as described above.

The cDNA clones were sequenced using an Applied Biosystems 373A automated DNA sequencer and Applied Biosystems Taq DyeDeoxy™ Terminator cycle sequencing kits following the manufacture's instructions. In some instances, sequences were obtained using [^{35}S]dATP (Amersham) and Sequenase™ kits from U.S. Biochemicals following the manufacturer's instructions. Both strands of the cDNA clone 44 were sequenced by using synthetic oligonucleotides as primers. The sequence of the most 5' 350 nt was determined in seven independent cDNA clones. The resultant clone demonstrated the pattern shown in Figure 30 (NDF).

EXAMPLE 9

Other Possible Splicing Variants

Alignment of the deduced amino acid sequences of the cDNA clones and PCR products of the bovine, and the published human (Fig. 31) and rat sequences show a high level of similarity, indicating that these sequences are derived from homologous genes within the three species. The variable number of messenger RNA transcripts detectable at the cDNA/PCR product level is probably due to extensive tissue-specific splicing. The patterns obtained and shown in Figure 30 suggests that other splicing variants exist. A list of probable splicing variants is indicated as followed. Many of these variants can be obtained by coding segment specific probing of cDNA libraries derived from different tissues. Alternatively, the variants can be assembled from specific (excised from) cDNA clones, PCR products or genomic DNA regions via cutting and splicing techniques known to one skilled in the art. These variant sequences can be expressed in recombinant systems and the recombinant products can be assayed to determine their

level of Schwann cell mitogenic activity as well as their ability to bind and activate the $p185^{erbB2}$ receptor.

EXAMPLE 10

Functional elements of GGF

The deduced structures of family of GGF sequences indicate that the longest forms (as represented by GGF2BPP4) encode transmembrane proteins where the extracellular part contains a domain which resembles epidermal growth factor (see Carpenter and Wahl in Peptide Growth Factors and Their Receptors I pp. 69-133, Springer-Verlag, NY 1991). The positions of the cysteine residues in coding segments C and C/D or C/D' peptide sequence are conserved with respect to the analogous residues in the epidermal growth factor (EGF) peptide sequence. This suggests that the extracellular domain functions as a receptor recognition and biological activation sites. Several of the variant forms lack the H, K, and L coding segments and thus may be expressed as secreted, diffusible biologically active proteins. Likely structures are shown in Figure 35.

Membrane bound versions of this protein may induce Schwann cell proliferation if expressed on the surface of neurons during embryogenesis or during nerve regeneration (where the surfaces of neurons are intimately associated with the surfaces of proliferating Schwann cells).

Secreted (non membrane bound) GGF's may act as classically diffusible factors which can interact with Schwann cells at some distance from their point of secretion. An example of a secreted GGF is the protein encoded by GGF2HBS5 (see example 6)

Other GGFs such as that encoded by GGF2BPP5 seem to be non-secreted (see example 6). These GGFs may be injury response forms which are released as a consequence of tissue damage.

EXAMPLE 11

Splicing variants with antiproliferative action

One particular splicing variant (GGF2BPP1) is described in Example 4. GGF2BPP1 is a truncated gene product which is generated by reading past the coding segment A splice junction into the adjoining genomic sequence. This represents coding segment A' in Figure 31. The transcript ends near to a canonical AATAAA polyadenylation sequence. This splicing variant contains regions F, E, B and A'. Other possible variants of this may lack region E (F, B, A'). As described in Example 10 regions C, C/D, or C/D' are homologous to EGF and are most likely to be the sites which are responsible for biological activity. GGF2BPP1 could retain receptor binding activity yet lack the ability to activate the receptor. Such a ligand would function as an antagonist since it would compete with active GGF/p185 *erbB2* ligands (eg. GGF2BPP5) for receptor binding. Other splicing variants such as those containing region E may also function as antagonists as described above. The presence of an extra domain such as that which is encoded by region E may result in structural differences which would interfere with biological activity following receptor binding. GGF2BPP2 may also be an inhibitor molecule. The presence of region C/D' in addition to region C/D in GGF2BPP2 adds sequence to the EGF related region which could potentially result in a protein which lacks biological activity. GGF2HBS11 is another potential inhibitor molecule. This clone was isolated from a human brainstem library using the same methods and probes as described in Example 6 for the isolation of GGF2HBS5. The GGF2HBS11 clone contains a portion of region E which is flanked by new sequence which is not contained in any other known region. The lack of region C, C/D or C/D' suggests that GGF2HBS11 would also lack biological activity.

EXAMPLE 12

Purification of antiproliferative factors from Recombinant Cells

In order to obtain antiproliferative factors to assay for biological activity, the proteins can be overproduced using cloned DNA. Several approaches can be used. A recombinant E. coli cell containing the sequences described in example 11 can be constructed. Expression systems such as pNH8a (Stratagene, Inc.) can be used for this purpose by following manufacturers procedures. Alternatively, these sequences can be inserted in a mammalian expression vector and an overproducing cell line can be constructed. As an example, for this purpose DNA encoding GGF2BPP1 can be expressed in COS cells or can be expressed in Chinese hamster ovary cells using the pMSXND expression vector (Lee and Nathans, J. Biol. Chem. 263, 3521-3527, (1981)). This vector containing GGF DNA sequences can be transfected into host cells using established procedures.

Transient expression can be examined or G418-resistant clones can be grown in the presence of methotrexate to select for cells that amplify the DHFR gene (contained on the pMSXND vector) and, in the process, co-amplify the adjacent protein encoding sequence. Because CHO cells can be maintained in a totally protein-free medium (Hamilton and Ham, in Vitro 13, 537-547 (1977)), the desired protein can be purified from the medium. Western analysis using the antisera produced in Example 9 can be used to detect the presence of the desired protein in the conditioned medium of the overproducing cells.

The desired protein can be purified from the E. coli lysate or the CHO cell conditioned medium using the types of procedures described in Example 1. The protein may be assayed at various points in the procedure using a Western blot assay.

EXAMPLE 13

Design and assay of antiproliferative factors

As indicated above and in Figs. 35 and 39-45, the GGF coding segments include regions with EGF-like homology. These EGF-like domains can be required for the activation of mitogenesis in the binding reaction between GGF ligands containing such domains and the erbB2 receptor. Comparisons of naturally occurring products of the GGF coding sequences which confer mitogenic activity versus those which confer antiproliferative activity, as disclosed above, provide additional support for this. Consequently, preferred antiproliferative factors are those which lack these EGF-like domains. Antiproliferative factors designed in this manner will lack all or part of the C, C/D, or C/D' coding segments. Examples of such factors likely to have antiproliferative activity using this design strategy are shown in Fig. 37 and described in the summary of the invention.

The recombinant proteins produced in Example 12 using the criterion described above may be assayed as described hereafter. The Schwann cell mitogenic assay described herein may be used to assay the expressed product of the full length clone or any biologically active portions thereof. Any member of the family of splicing variant complementary DNA's derived from the GGF gene (including the Heregulins) can be expressed in this manner and assayed in the Schwann cell proliferation assay by one skilled in the art. Antiproliferative activity in the GGF assay can be examined by a competition assay (Chan et al., Science 254:1383 (1991)). Varying concentrations of recombinant antiproliferative GGF variants (such as GGF2BPP1) can be added to Schwann cell cultures in the presence of GGF. The extent of antiproliferative activity can be measured by comparing mitogenic activity of the cultures to controls treated only with GGF. This will

provide a measure of dose dependent inhibition. The specificity of the response can be measured by examining the effect of varying concentrations of antiproliferative factor on the mitogenic activity of other growth factors and their target cells (e.g. EGF). Antiproliferative activity of recombinant GGF variants can also be examined in breast tumour cells. Cell lines such as SK-BR-3 which proliferate in response to GGF's/$p185^{erbB2}$ ligands can be assayed in a similar manner to that described above for Schwann cells.

Crosslinking studies can be performed to determine whether I^{125} labelled GGF variants, which show antiproliferative activity (as described above), bind to the erbB2 receptor (Chan et al., Science 254:1383 (1991)). Binding can be demonstrated by immunoprecipitation of the cross-linked protein with an antibody to the erbB2 receptor.

What is claimed is:

Claims

1. A DNA sequence encoding a polypeptide synthesized by the plasmid pGGF2HBS11 deposited with the A.T.C.C. November 6, 1992 (A.T.C.C. Deposit No. 75347).

2. A polypeptide encoded by pGGF2HBS11 deposited with the A.T.C.C. November 6, 1992 (A.T.C.C. Deposit No. 75347).

3. A polypeptide comprising the peptide encoded by the E sequence (SEQ ID Nos. 137 and 163) and at least a portion of the peptide encoded by the brain-derived DNA sequences flanking the E encoding sequence on clone pGGF2HBS11, ATCC Deposit No. 75347.

4. The polypeptide of claim 3, wherein said peptide encoded by the E sequence (SEQ ID Nos. 137 and 163) has a deletion of the 48 N-terminal amino acids and wherein said E-flanking peptide sequences contain between 20 and 100 N-terminal amino acids and between 30 and 50 C-terminal amino acids encoded by said clone.

5. The polypeptide of claim 3, wherein said peptide encoded by the E sequence (SEQ ID Nos. 137 and 163) has a deletion of the 48 N-terminal amino acids and wherein said E-flanking peptide sequences contain between 25 and 70 N-terminal amino acids and between 30 and 45 C-terminal amino acids encoded by said clone.

6. A method for inhibiting proliferation of a cell, said method comprising contacting said cell with a polypeptide defined by the formula

VYBAZWX

wherein VYBAZWX is composed of the polypeptide segments shown in Figure 31 (SEQ ID Nos. 136-139, 141-147, 160, 161, and 163); wherein V comprises F, or is absent; wherein Y comprises polypeptide segment E, or is absent; wherein Z comprises polypeptide segment G or is absent; wherein W comprises C or is absent; and wherein X comprises polypeptide segments C/D HKL, C/D H, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, C/D C/D' D' HKL, H, HL, or HKL.

7. A method for inhibiting proliferation of a cell, said method comprising contacting said cell with a polypeptide comprising FBA polypeptide segments, FBA' polypeptide segments, EBA polypeptide segments, EBA' polypeptide segments, FEBA polypeptide segments, or FEBA' polypeptide segments having the amino acid sequences corresponding to polypeptide segments shown in Figure 31 (SEQ ID Nos. 136-140, 163, 168).

8. A method of inhibiting proliferation of a cell, said method comprising contacting said cell with a polypeptide of claims 1-5.

9. A method of inhibiting proliferation of a cell, said method comprising contacting said cell with a compound which specifically binds the $p185^{erbB2}$ receptor of said cell.

10. The method of any one of claims 6-9, wherein said cell is a cell of the nervous system.

11. The method of claim 10, wherein said cell is a glial cell.

12. The method of claim 11, wherein said cell is a Schwann cell.

13. A method of any one of claims 6-9 wherein said cell is a cancer cell.

14. A method of claim 13, wherein said cell is an adenocarcinoma cell.

15. The method of any one of claims 6-9, wherein said method is used in the treatment or prophylaxis of a nervous disease or disorder.

16. The method of any one of claims 6-9, wherein said cell is a cell in a mammal and said contacting is carried out by administering said peptide to said mammal for the prophylaxis or treatment of a pathophysiological condition in said mammal in which said condition involves said cell.

17. The method of any one of claims 6-9, wherein said method is used for the treatment or prophylaxis of a demyelination disease or disorder.

18. A method as claimed in claim 16, wherein said condition involves a disease of cell proliferation, such as a tumor or peripheral nerve damage caused by a cell tumor.

19. The method of any one of claims 6-9, wherein said cell is in a mammal and said contacting is carried out by administering said peptide to said mammal for the prophylaxis or treatment of a condition which involves a tumor of said cell, for example, neurofibromatosis, malignant Schwannomas or neurofibrosarcomas.

20. The method of any one of claims 6-9, wherein said cell is in a mammal and said contacting is carried out by administering said peptide to said mammal for prophylaxis or treatment of a condition which involves a meningioma, a bilateral acoustic neuroma, a astrocytoma, a retinoblastoma, a neuroglioma, a neuroblastoma, or a glioma.

21. A method for producing an antibody specific for a polypeptide, said method comprising

i) immunizing a mammal with a polypeptide selected from the group consisting of a polypeptide defined by the formula

VYBAZWX

wherein VYBAZWX is composed of the polypeptide segments shown in Figure 31 (SEQ ID Nos. 136-139, 141, 146, 147, 160, 161, and 163); wherein V comprises F, or is absent; wherein Y comprises polypeptide segment E, or is absent; wherein Z comprises polypeptide segment G or is absent; wherein W comprises C or is absent; and wherein X comprises polypeptide segments H, HK, or HKL, and

ii) purifying said antibody from tissue of said animal, or from a hybridoma made using said tissue.

22. A method for producing an antibody specific for a polypeptide, said method comprising

i) immunizing a mammal with a polypeptide selected from the group consisting of polypeptides defined by the formulas FBA, FBA′, EBA, FEBA, FEBA′, or EBA′, wherein F, E, B, A and A′ segments are defined by the amino acid sequences shown in Figure 31 (SEQ ID Nos. 136-140, 163, 168) and

ii) purifying said antibody from tissue of said animal, or from a hybridoma made using said tissue.

23. A method for detecting, in a sample, the presence of a molecule capable of binding to a receptor which binds to a polypeptide selected from the group consisting of polypeptides defined by the formula

VYBAZWX

wherein VYBAZWX is composed of the polypeptide segments shown in Figure 31 (SEQ ID Nos. 136-139, 141, 146, 147, 160, 161, and 163); wherein V comprises F, or is absent; wherein Y comprises polypeptide segment E, or is absent; wherein Z comprises polypeptide segment G or is absent; wherein W comprises C or is absent; and wherein X comprises polypeptide segments H, HK, or HKL,

said method comprising the steps of

i) contacting said sample with said polypeptide together with said receptor, and

ii) detecting competitive inhibition of the binding of said polypeptide to said receptor as an indication of the presence of a receptor binding molecule in said sample.

24. A method for detecting, in a sample, the presence of a molecule capable of binding to a receptor which binds to a polypeptide selected from the group consisting of polypeptides defined by the formulas FBA, FBA', EBA, FEBA, FEBA', or EBA', wherein F, E, B, A and A' segments are defined by the amino acid sequences shown in Figure 31 (SEQ ID Nos. 136-140, 163, 168), said method comprising the steps of

i) contacting said sample with said polypeptide together with said receptor, and

ii) detecting competitive inhibition of the binding of said polypeptide to said receptor as an indication of the presence of a receptor binding molecule in said sample.

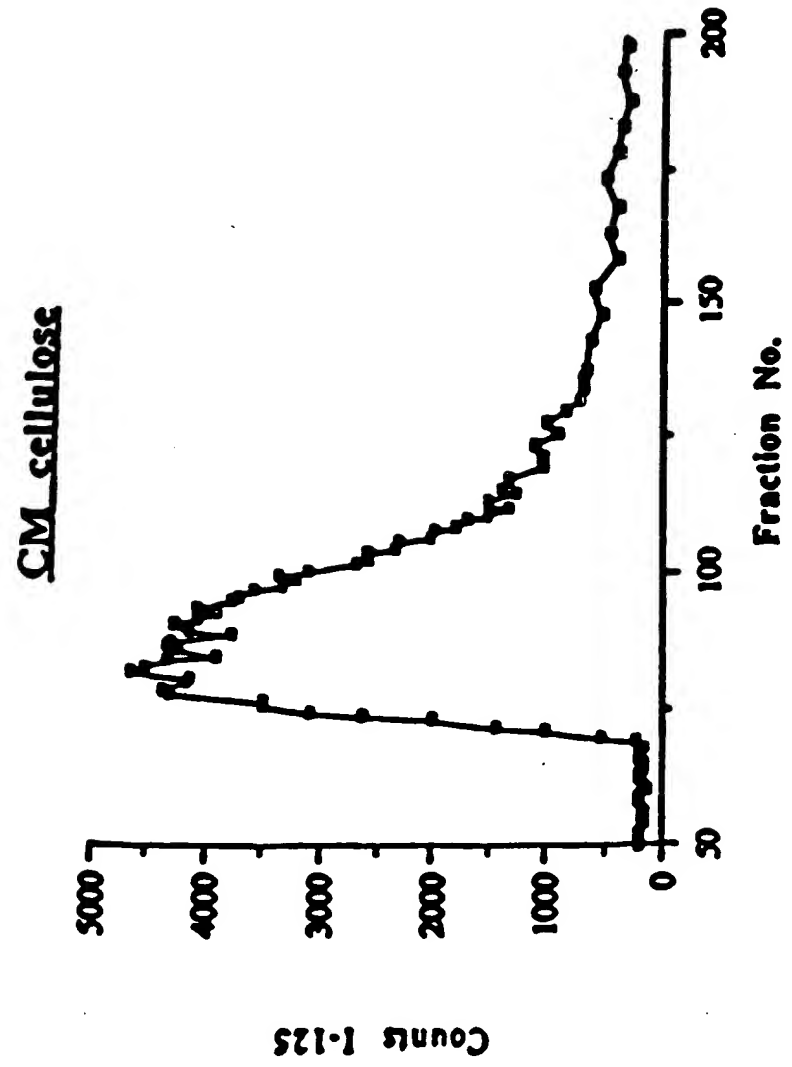

CM cellulose
Counts I-125
5000
4000
3000
2000
1000
0
50
100
150
200
Fraction No.

FIGURE 1

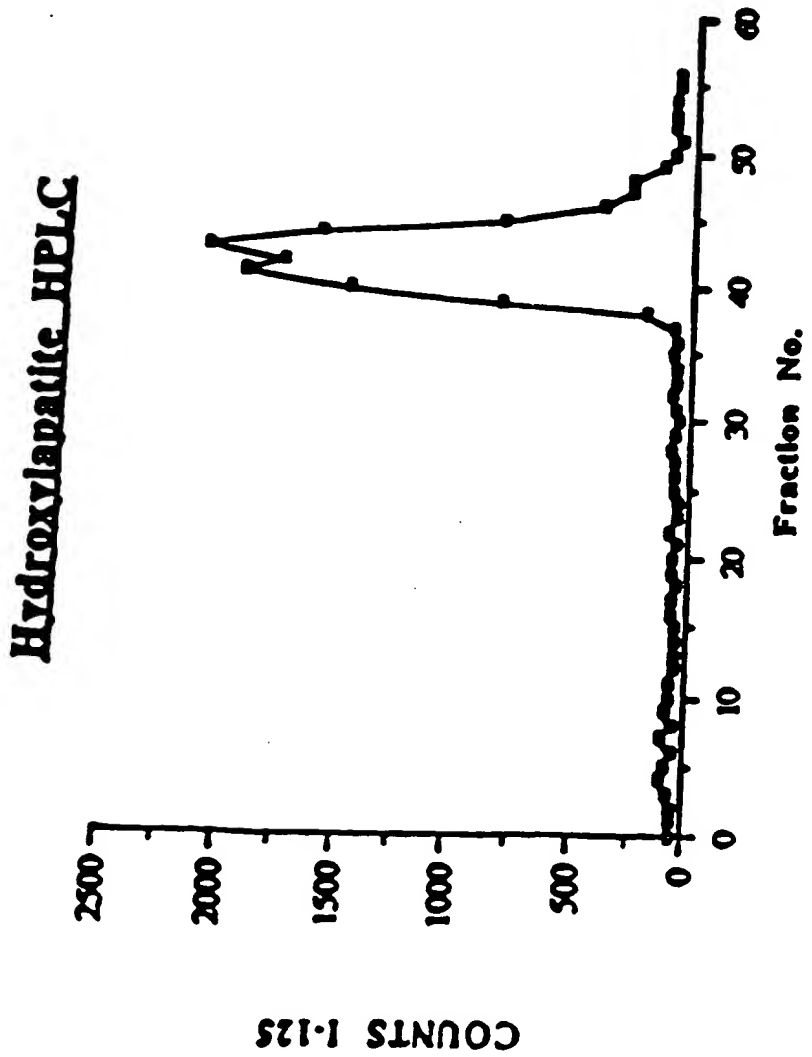

Hydroxylapatite HPLC
COUNTS 1.125
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
Fraction No.

FIGURE 2

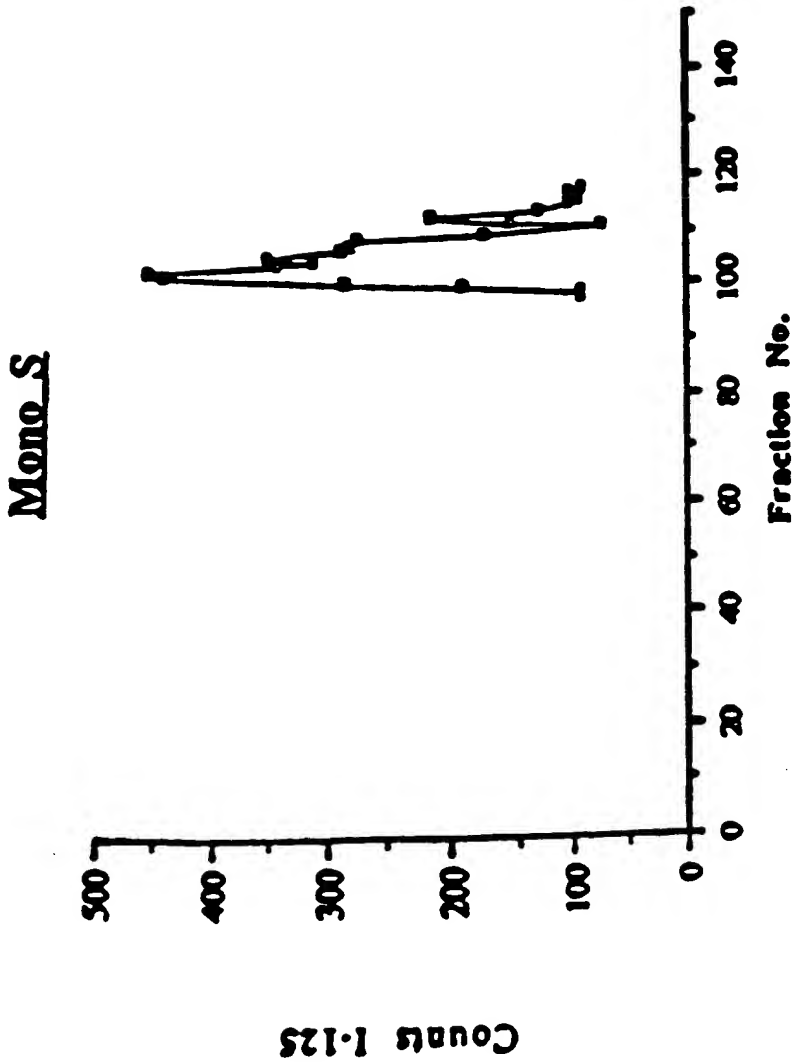

Mono S
Fraction No.
0
20
40
60
80
100
120
140
100
200
300
400
500
Counts 1.125

FIGURE 3

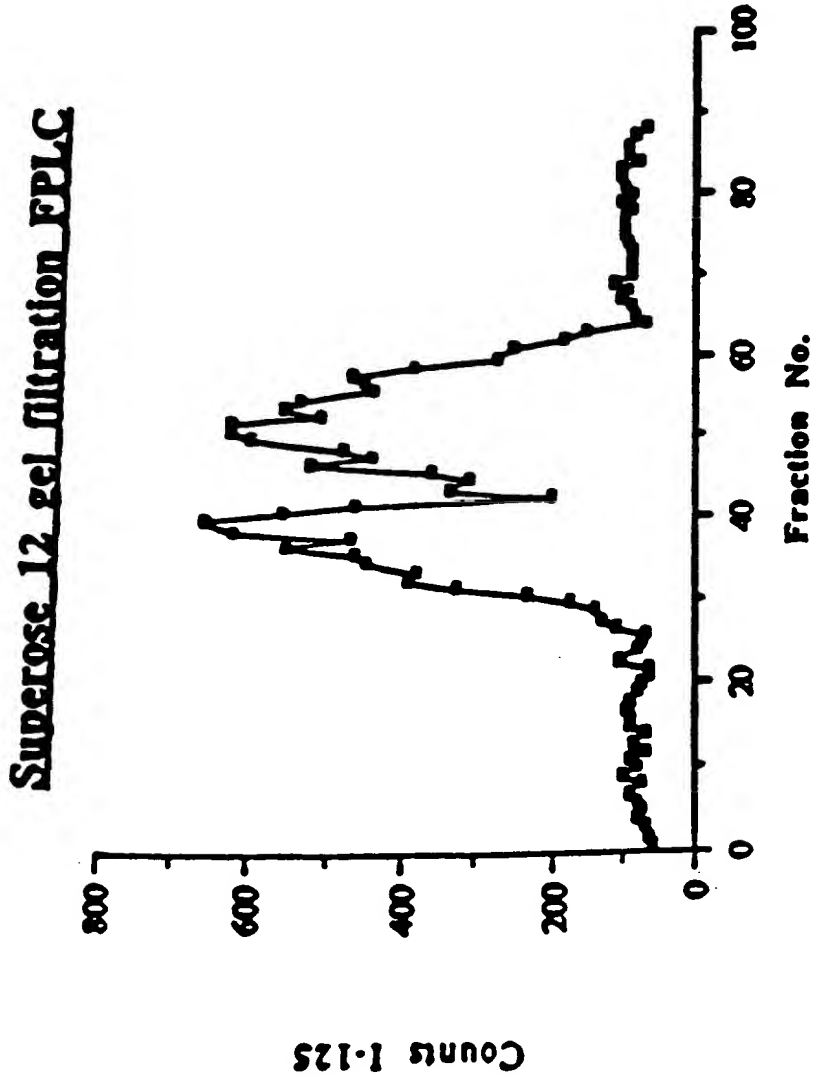

Superose 12 gel filtration FPLC
Counts I-125
800
600
400
200
0
0
20
40
60
80
100
Fraction No.

FIGURE 4

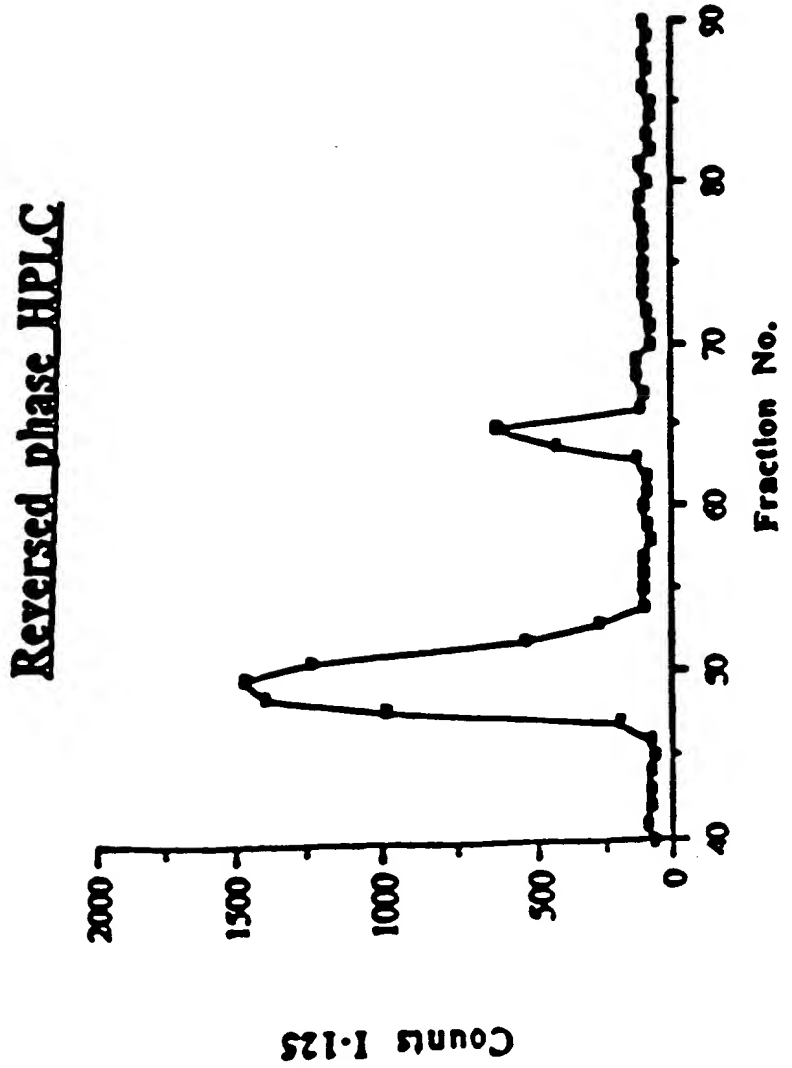

Reversed phase HPLC
Counts I-125
2000
1500
1000
500
0
40
50
60
70
80
90
Fraction No.

FIGURE 5

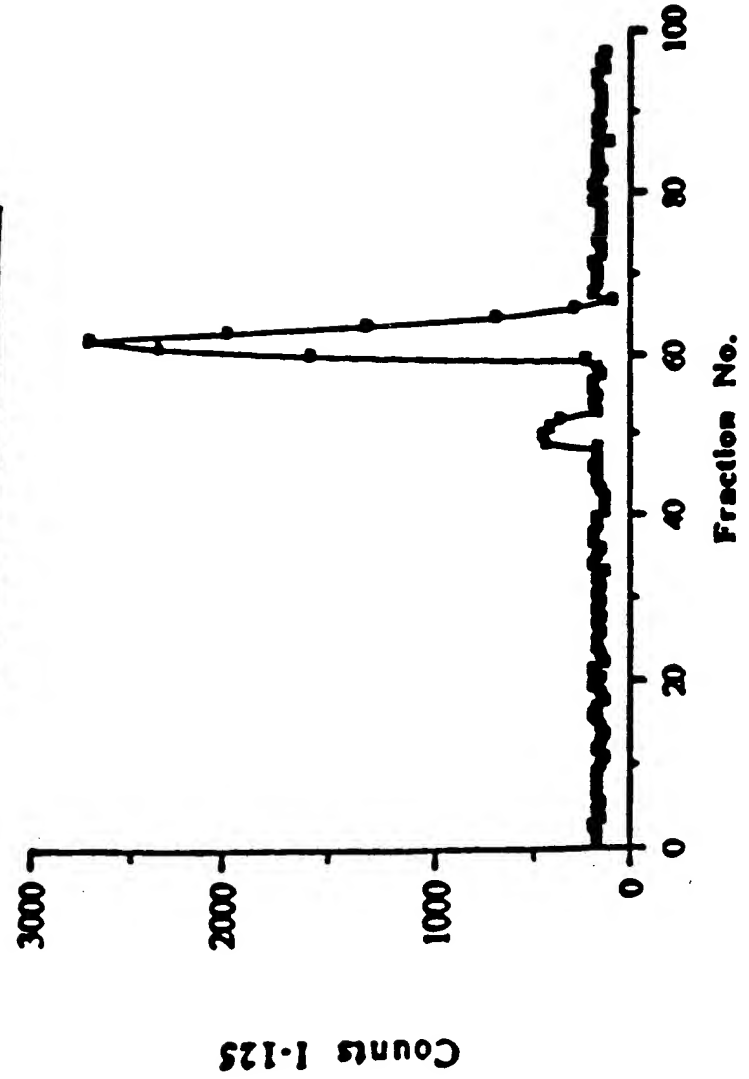

Reversed phase HPLC
Counts I-125
3000
2000
1000
0
0
20
40
60
80
100
Fraction No.

FIGURE 6

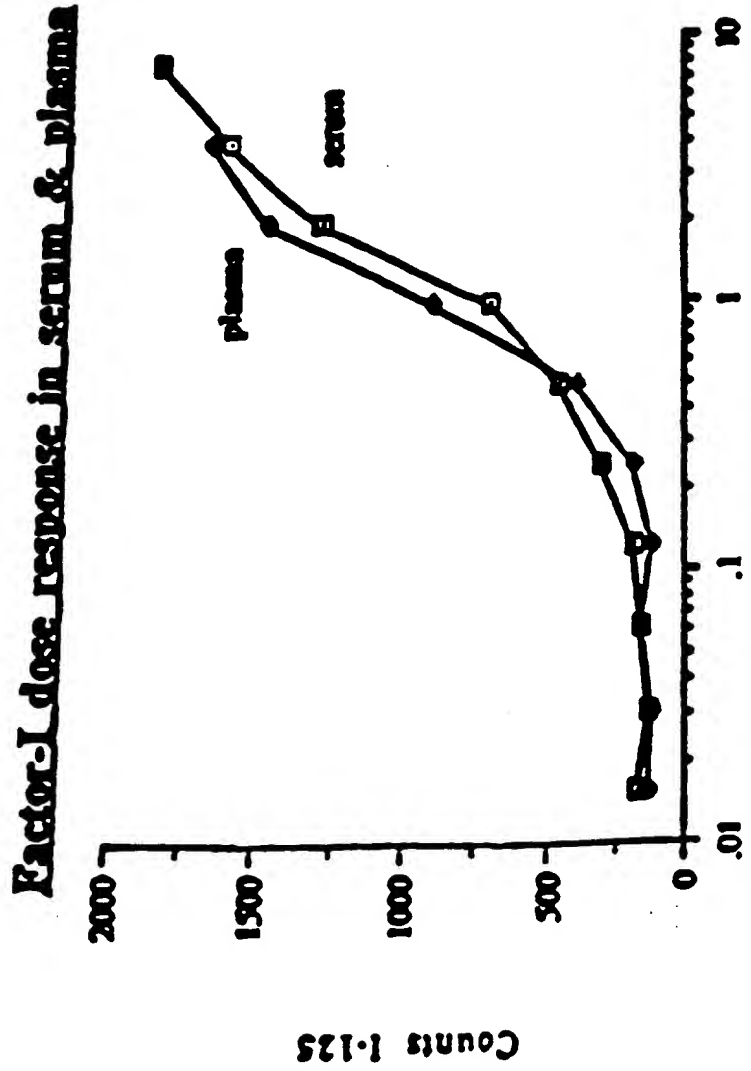

Factor-I dose response in serum & plasma
plasma
serum
2000
1500
1000
500
0
.01
.1
1
10
uL/well
Counts I-125

FIGURE 7

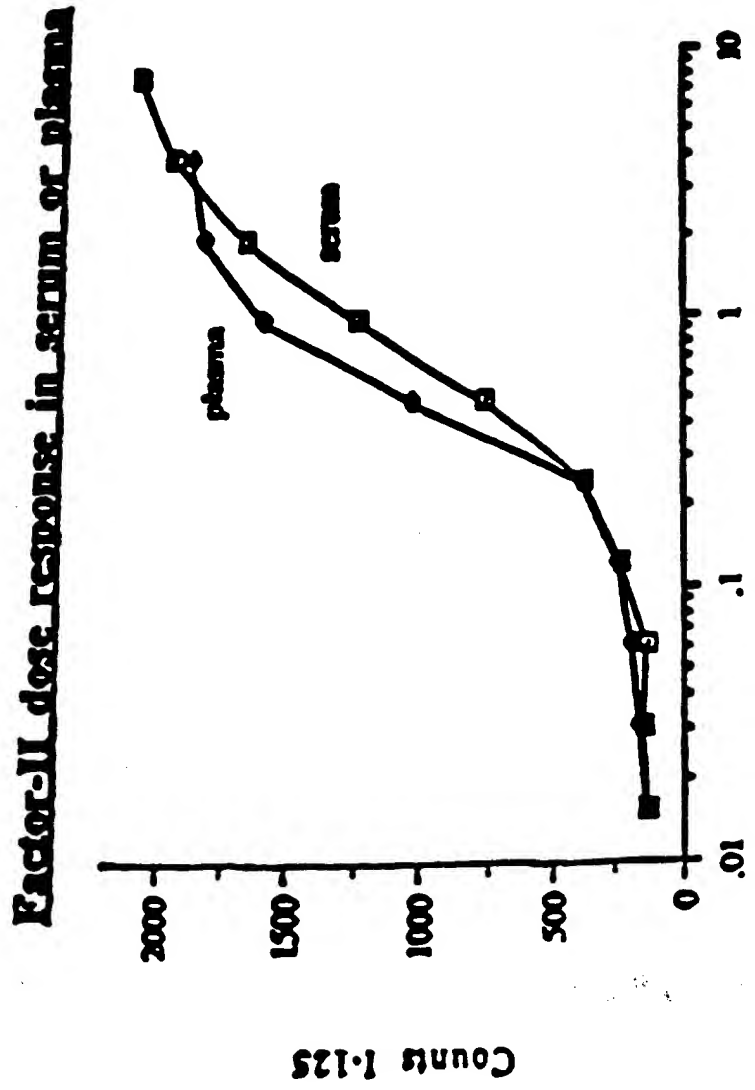

Factor-U dose response in serum or plasma
Counts I-125
2000
1500
1000
500
0
.01
.1
1
10
uL/well
plasma
serum

FIGURE 8

FIGURE 9

	N-terminus		
NF-1	1	F K G D A H T E (SEQ ID NO: 1)	
	Trypsin peptides		
NF-1	02	K/R A S L A D E Y E Y M X K * (SEQ ID NO: 2	
NF-1	03	K/R T E T S S S G L X L K * (SEQ ID NO: 3)	
NF-1	04	K/R K L G E M W A E (SEQ ID NO: 4)	
NF-1	05	K/R L G E K R A (SEQ ID NO: 5)	HMG-1
NF-1	06	K/R I K S E H A G L S I G D T A K * (SEQ ID NO: 6)	HMG-17
NF-1	07	K/R A S L A D E Y E Y M R K * (SEQ ID NO: 7)	HMG-2
NF-1	08	K/R I K G E H P G L S I G D V A K * (SEQ ID NO: 8)	HMG-1
NF-1	09	K/R M S E Y A F F V Q T X R * (SEQ ID NO: 9)	HMG-2
NF-1	10	K/R S E H P G L S I G D T A K * (SEQ ID NO: 10)	HMG-1
NF-1	11	K/R A G Y F A E X A R * (SEQ ID NO: 11)	
NF-1	12	K/R K L E F L X A K * (SEQ ID NO: 12)	
NF-1	13	K/R T T E M A S E Q G A (SEQ ID NO: 13)	
NF-1	14	K/R A K E A L A A L K * (SEQ ID NO: 14)	
NF-1	15	K/R F V L Q A K K * (SEQ ID NO: 15)	
NF-1	16	K/R L G E M W (SEQ ID NO: 16)	HMG-1
	Protease V8 peptides		
NF-1	17	E T Q P D P G Q I L K K V P M V I G A Y T (SEQ ID NO: 169)	
NF-1	18	E Y K C L K F K W F K K A T V M (SEQ ID NO: 17)	
NF-1	19	E A K Y F S K X D A (SEQ ID NO: 18)	U1-alpha
NF-1	20	E X K F Y V P (SEQ ID NO: 19)	
NF-1	21	E L S F A S V R L P G C P P G V D P M V S F P V A L	U1-beta (SEQ ID N

Figure 10

A

OGF-I 01	F K G D A H T E	(SEQ ID NO: 21)
OGF-I 02	A S L A D E Y E Y M X K	(SEQ ID NO: 22)
OGF-I 03	T E T S S S G L X L K	(SEQ ID NO: 23)
OGF-I 07	A S L A D E Y E Y M R K	(SEQ ID NO: 24)
OGF-I 11	A G Y F A E X A R	(SEQ ID NO: 25)
OGF-I 13	T T E M A S E Q G A	(SEQ ID NO: 26)
OGF-I 14	A K E A L A A L K	(SEQ ID NO: 27)
OGF-I 15	F V L Q A K K	(SEQ ID NO: 28)
OGF-I 17	E T Q P D P G Q I L K K V P M V I G A Y T	(SEQ ID NO: 29)
OGF-I 18	E Y K C L K F K W F K K A T V M	(SEQ ID NO: 30)

B

OGF-I 20	E X K F Y V P	(SEQ ID NO: 31)
OGF-I 12	K L E F L X A K	(SEQ ID NO: 32)

Figure 11

Trypsin peptides

GF-II 01	K/R V H Q V W A A K•		(SEQ ID NO: 45)
GF-II 02	K/R Y I F F M E P E A X S S G		(SEQ ID NO: 46)
GF-II 03	K/R L G A W G P P A F P V X Y		(SEQ ID NO: 47)
GF-II 04	K/R W F V V I E G K•		(SEQ ID NO: 48)
GF-II 05	K/R A L A A A G Y D V E K•	Histone H1	(SEQ ID NO: 164)
GF-II 06	K/R L V L R•		(SEQ ID NO: 165)
GF-II 07	K/R X X Y P G Q I T S N	Trypsin	(SEQ ID NO: 166)
GF-II 08	K/R A S P V S V G S V Q E L V Q R•		(SEQ ID NO: 49)
GF-II 09	K/R V C L L T V A A L P P T		(SEQ ID NO: 50)
GF-II 10	K/R D L L L X V		

Lysyl Endopeptidase-C peptides

GF-II 11	K V H Q V W A A K•	(SEQ ID NO: 51)
GF-II 12	K A S L A D S G E Y M X K•	(SEQ ID NO: 52)

Figure 12

A

GGF-II 01	VHQVWAAK	(SEQ ID NO: 45)
GGF-II 02	YIFFMEPEAXSSG	(SEQ ID NO: 46)
GGF-II 03	LGAWGPPAFPVXY	(SEQ ID NO: 47)
GGF-II 04	WFVVIEGK	(SEQ ID NO: 48)
GGF-II 08	ASPVSVGSVQELVQR	(SEQ ID NO: 49)
GGF-II 09	VCLLTVAALPPT	(SEQ ID NO: 50)
GGF-II 11	KVHQVWAAK	(SEQ ID NO: 51)
GGF-II 12	KASLADSGEYMXK	(SEQ ID NO: 52)

B Novel Factor II Peptides - others

GGF-II 10	DLLLXV	(SEQ ID NO: 53)

Comparison of BrdU-ELISA and [125 I]UdR counting method for the DNA synthesis assay in Schwann cell cultures

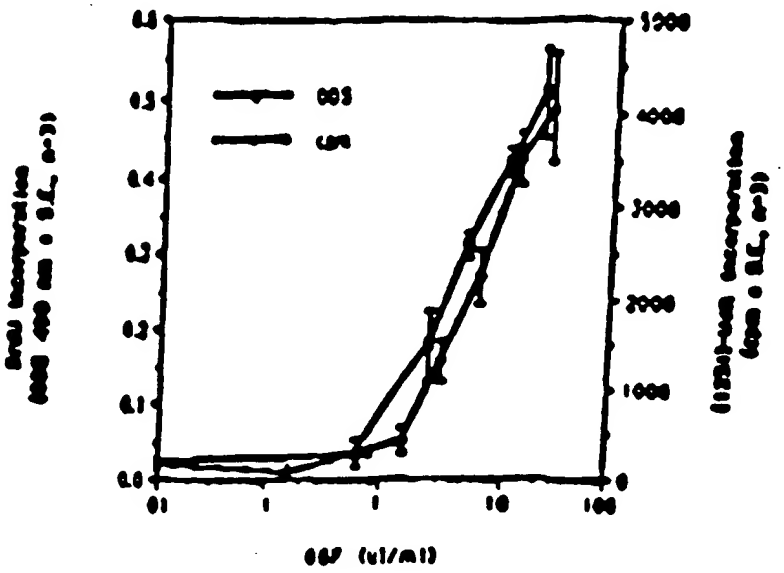


Fig.13

Comparison of Br-UdR immunoreactivity and Br-UdR labelled cell number

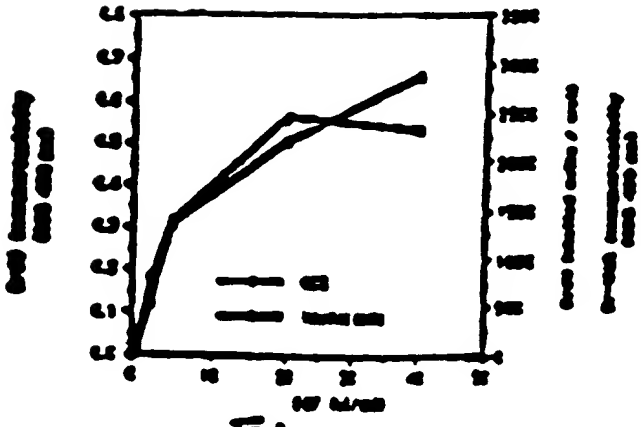


Fig.14a

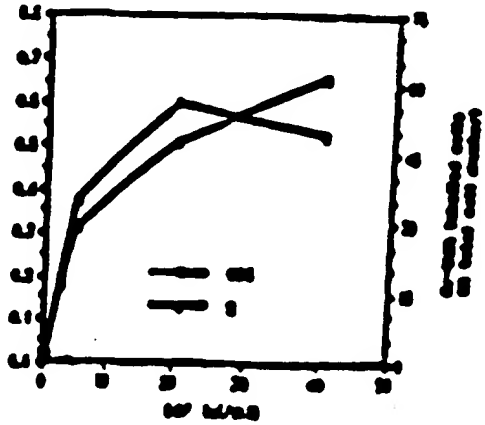


Fig.14b.

Mitogenic response of rat sciatic nerve Schwann cell to GGFs

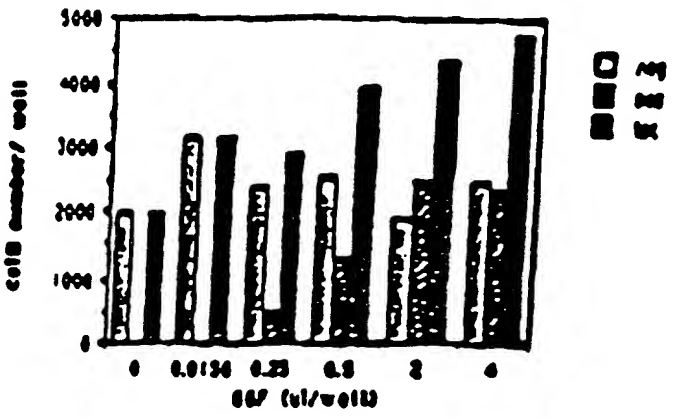


Fig # 15

DNA synthesis in rat sciatic nerve Schwann cells and 3T3 fibroblasts in the presence of GGFs

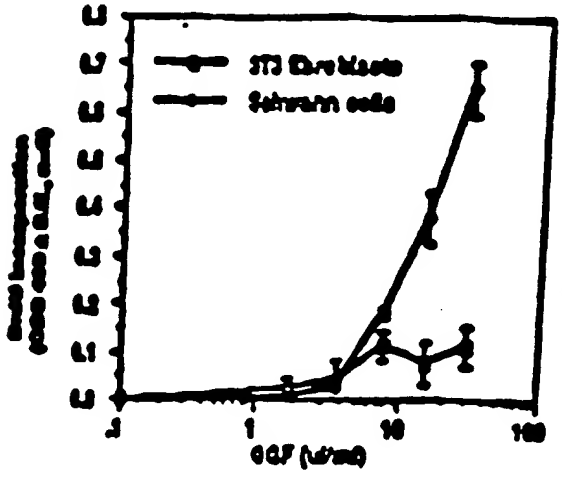


Fig. # 16.

Mitogenic response of BHK 21 C13 cells to FCS and GGFs

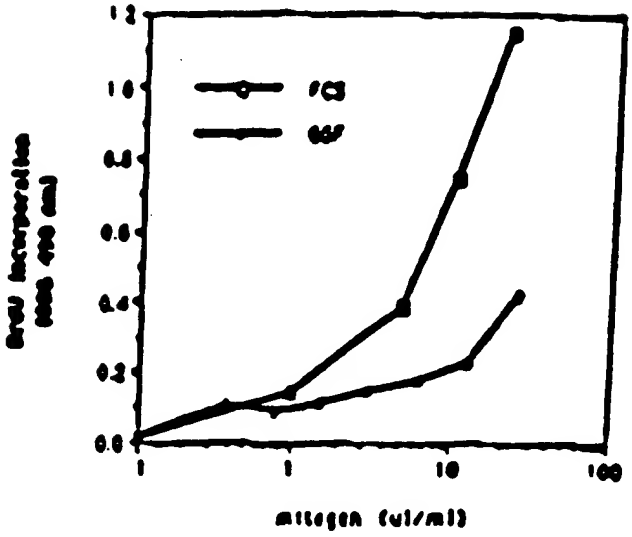


Fig. 17

Survival and proliferation of BHK21 C13 cell microcultures after 48 hours in presence of GGFs

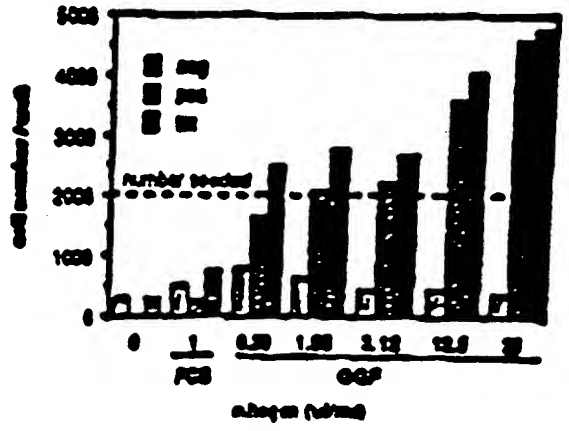


Fig. 18.

Mitogenic response of C6 cells to FCS

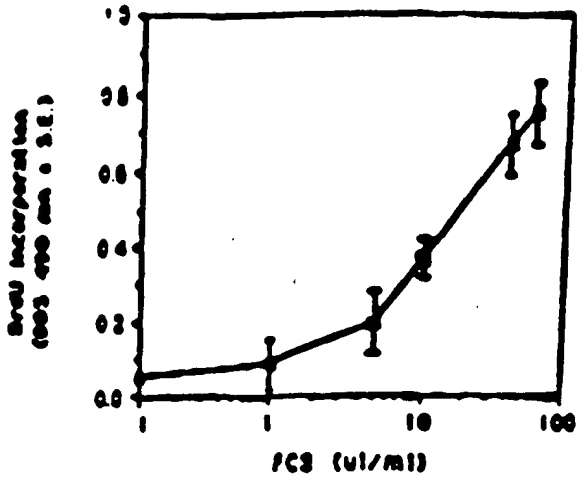


Fig 19.

Mitogenic response of C6 cells to aFGF and GGFs

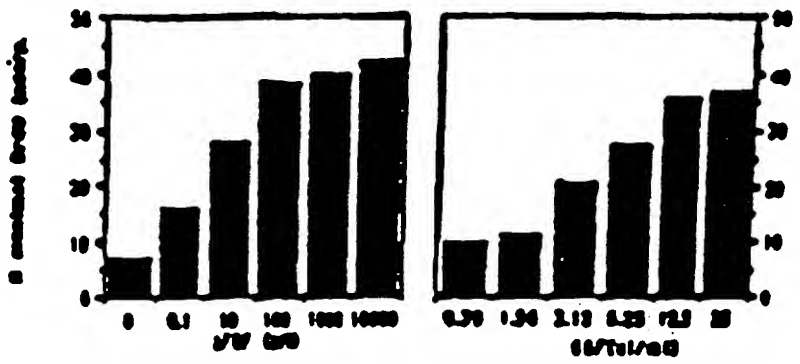


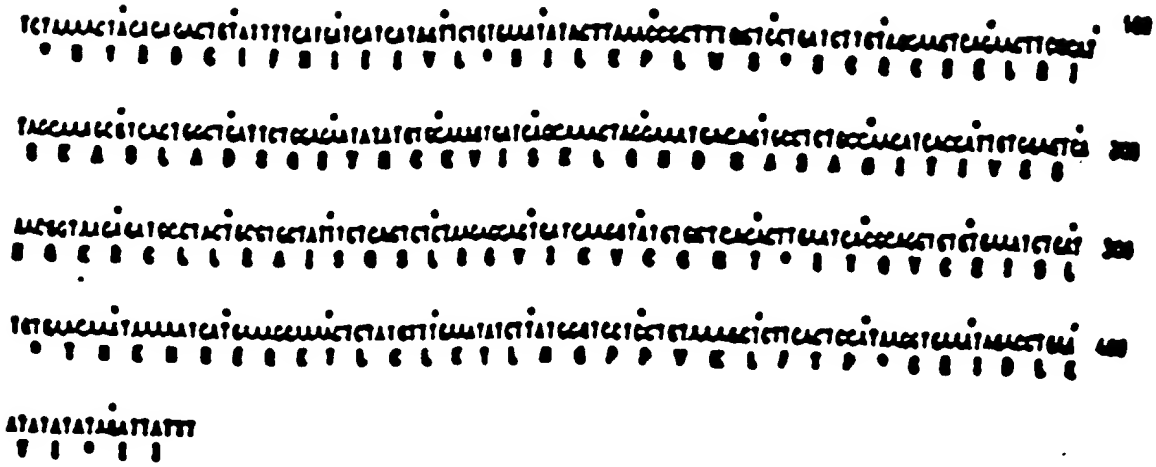
Fig 20

FIGURE 21

DEGENERATE OLIGONUCLEOTIDE PROBES FOR FACTOR I AND FACTOR II

Oligo	Sequence	Peptide	
535	TTYAARGGNGAYGCNCAYAC!	GGFI-1	(SEQ ID NO: 54)
536	CATRTAYTCRTAYTCRTCNGC!	GGFI-2	(SEQ ID NO: 55)
537	TGYTCNGAYGCCATYTCNGT!	GGFI-13	(SEQ ID NO: 56)
538	TGYTCRCTNGCCATYTCNGT!	GGFI-13	(SEQ ID NO: 57)
539	CCDATNACCATNGGNACYTT!	GGFI-17	(SEQ ID NO: 58)
540	GCNGCCCAYACYTGRTGNAC!	GGFII-1	(SEQ ID NO: 59)
541	GCYTCNGGYTCCATRAARAA!	GGFII-2	(SEQ ID NO: 60)
542	CCYTCDATNACNACRAACCA!	GGFII-4	(SEQ ID NO: 61)
543	TCNGCRAARTANCCNGC!	GGFI-11	(SEQ ID NO: 62)
544	GCNGCNAGNGCYTCYTTNGC!	GGFI-14	(SEQ ID NO: 63)
545	GCNGCYAANGCYTCYTTNGC!	GGFI-14	(SEQ ID NO: 64)
546	TTYTTNGCYTGNAGNACRAA!	GGFI-15	(SEQ ID NO: 65)
551	TTYTTNGCYTGYAANACRAA!	GGFI-15	(SEQ ID NO: 66)
568	TGNACNAGYTCYTGNAC!	GGFII-8	(SEQ ID NO: 67)
569	TGNACYAAYTCYTGNAC!	GGFII-8	(SEQ ID NO: 68)
609	CATRTAYTCNCCNGARTCNGC!	GGFII-12	(SEQ ID NO: 69)
610	CATRTAYTCNCCRCTRTCNGC!	GGFII-12	(SEQ ID NO: 70)
649	NGARTCNGCYAANGANGCYTT!	GGFII-12	(SEQ ID NO: 71)
650	NGARTCNGCNAGNGANGCYTT!	GGFII-12	(SEQ ID NO: 72)
651	RCTRTCNGCYAANGANGCYTT!	GGFII-12	(SEQ ID NO: 73)
652	RCTRTCNGCNAGNGANGCYTT!	GGFII-12	(SEQ ID NO: 74)
653	NGARTCNGCYAARCTNGCYTT!	GGFII-12	(SEQ ID NO: 75)
654	NGARTCNGCNAGRCTNGCYTT!	GGFII-12	(SEQ ID NO: 76)
655	RCTRTCNGCYAARCTNGCYTT!	GGFII-12	(SEQ ID NO: 78)
656	RCTRCTNGCNAGRCTNGCYTT!	GGFII-12	(SEQ ID NO: 79)
659	ACNACNGARATGGCTCCNGA!	GGFI-13	(SEQ ID NO: 80)
660	ACNACNGARATGGCAGTNGA!	GGFI-13	(SEQ ID NO: 81)
661	CAYCARGTNTGGGCNGCNAA!	GGFII-1	(SEQ ID NO: 82)
662	TTYGTNGTNATEGARGCNAA!	GGFII-4	(SEQ ID NO: 83)
663	AARGGNGAYGCNCAYACNGA!	GGFI-1	(SEQ ID NO: 84)
664	GARGCYTTNGCNGCYTTNAA!	GGFI-14	(SEQ ID NO: 85)
665	GTNGCNTCNGTNCARGARTT!	GGFII-8	(SEQ ID NO: 86)
666	GTNGCNAGYGTNCARGARTT!	GGFII-8	(SEQ ID NO: 87)
694	NACYTTYTTNARRATYTGNCC!	GGFI-17	(SEQ ID NO: 88)

Activin Beta Factor II Gene Sequence



(SEQ ID NO: 89)

FIGURE 22

PCR PRIMERS FOR FACTOR I AND FACTOR II FIGURE 23

Degenerate PCR primers

Oligo	Sequence	Peptide	
657	CCGAATTCTGCAGGARACNCARCCNGAYCCNGG!	GGFI-17	(SEQ ID NO: 90)
658	AAGGATCCTGCAGNGTRTANGCNCCRATNACCATNGG!	GGFI-17	(SEQ ID NO: 91)
667	CCGAATTCTGCAGGCNGAYTCNGGNGARTAYATG!	GGFII-12	(SEQ ID NO: 92)
668	CCGAATTCTGCAGGCNGAYATYGGNGARTAYAT!	GGFII-12	(SEQ ID NO: 93)
669	AAGGATCCTGCAGNNNCATRTAYTCNCCNGARTC!	GGFII-12	(SEQ ID NO: 94)
670	AAGGATCCTGCAGNNNCATRTAYTCNCCRRTRTC!	GGFII-12	(SEQ ID NO: 95)
671	CCGAATTCTGCAGCAYCARGTNTGGGCNGCNAA!	GGFII-1	(SEQ ID NO: 96)
672	CCGAATTCTGCAGATHTTYTTYATGGARCCNGARG!	GGFII-2	(SEQ ID NO: 97)
673	CCGAATTCTGCAGGGGGNCCNCCNGCNTTYCCNGT!	GGFII-3	(SEQ ID NO: 98)
674	CCGAATTCTGCAGTGGTTYGTNGTNATHGARGG!	GGFII-4	(SEQ ID NO: 99)
677	AAGGATCCTGCAGYTTNGCNGCCCANACYTGRTG!	GGFII-1	(SEQ ID NO: 100)
678	AAGGATCCTGCAGGCYTCNGGYTCCATRAARAA!	GGFII-2	(SEQ ID NO: 101)
679	AAGGATCCTGCAGACNGGRAANGCNGGNGGNCC!	GGFII-3	(SEQ ID NO: 102)
680	AAGGATCCTGCAGYTTNCCYTCDATNACNACRAAC!	GGFII-4	(SEQ ID NO: 103)
681	CATRTAYTCRTAYTCTCNGCAAGGATCCTGCAG!	GGFI-2	(SEQ ID NO: 104)
682	CCGAATTCTGCAGAARGGNGAYGCNCAYACNGA!	GGFI-1	(SEQ ID NO: 105)
683	GCNGCYAANGCYRCYTTNGCAAGGATCCTGCAG!	GGFI-14	(SEQ ID NO: 106)
684	GCNGCNAGNGCYTCYTTNGCAAGGATCCTGCAG!	GGFI-14	(SEQ ID NO: 107)
685	TCNGCRAARTANCCNGCAAGGATCCTGCAG!	GGFII-1	(SEQ ID NO: 108)

Unique PCR primers for Factor II

Oligo	Sequence	Comment	
711	CATCGATCTGCAGGCTGATTCTGGAGAATATATGTGCA!	3' RACE	(SEQ ID NO: 109)
712	AAGGATCCTGCAGCCACATCTGAGTCGACATCGATT!	3' RACE	(SEQ ID NO: 110)
713	CCGAATTCTGCAGTGATCAGCAAACTAGGAAATGACA!	3' RACE	(SEQ ID NO: 111)
721	CATCGATCTGCAGCCTAGTTTGCTGATCACTTTGCAC!	5' RACE	(SEQ ID NO: 112)
722	AAGGATCCTGCAGTATATTCTCCAAATCAGCCAGTG!	5' RACE; ANCHORED	(SEQ ID NO: 11
725	AAGGATCCTGCAGGCAGCAGTAGGCATCTCTTA!	EXON A	(SEQ ID NO: 114)
726	CCGAATTCTGCAGCAGAACTTGGCATTAGCAAAGC!	EXON A	(SEQ ID NO: 115)
771	CATCCCGGGATGAAGAGTCAGGAGTCTGTGGCA!	EXONS B+A	(SEQ ID NO: 116)
772	ATACCCGGGCTGCAGATGAGATTTCACACACCTGCG!		(SEQ ID NO: 117)
773	AAGGATCCTGCAGTTTGGAACCTGCCACAGACTCCT!	ANCHORED	(SEQ ID NO: 118)
776	ATACCCGGGCTGCAGATGAGATTTCACACACCTGCGTGA!	EXONS B+A	(SEQ ID NO: 119)

Summary of contiguous GGF-II cDNA structures and sequences

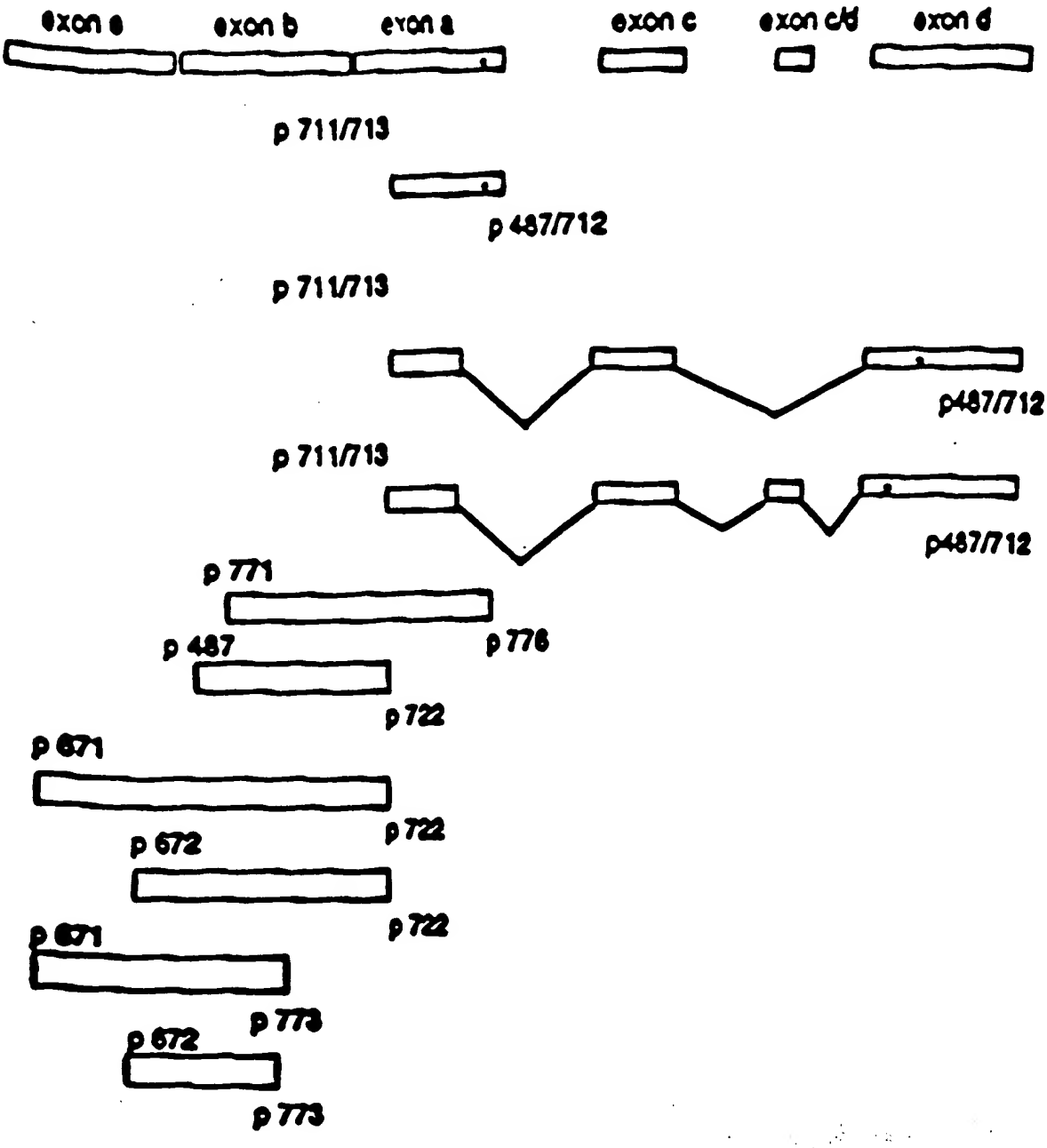
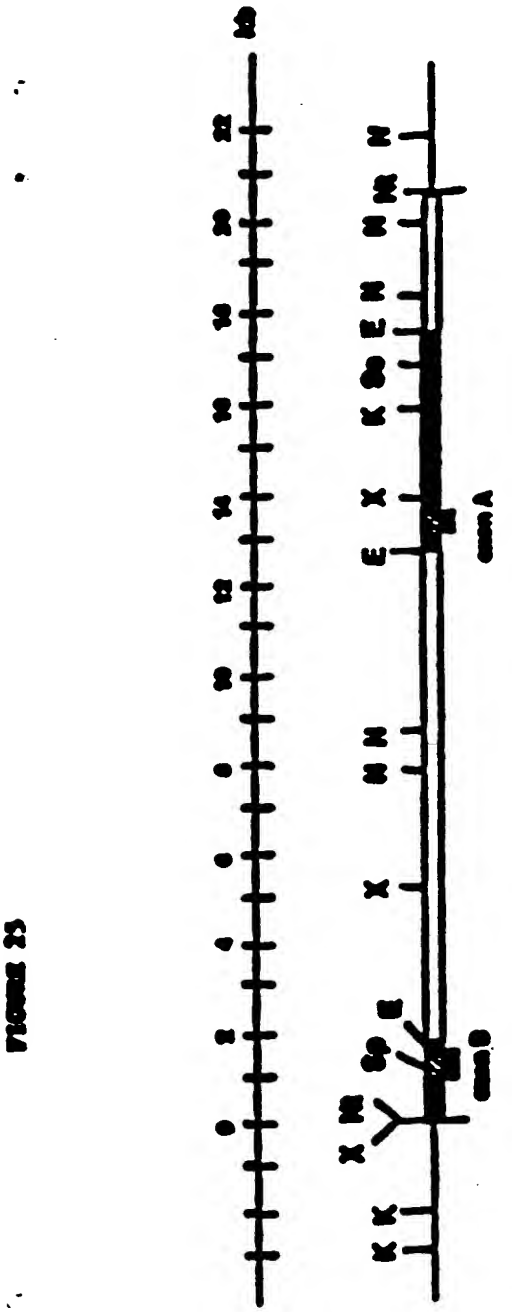


FIGURE 24



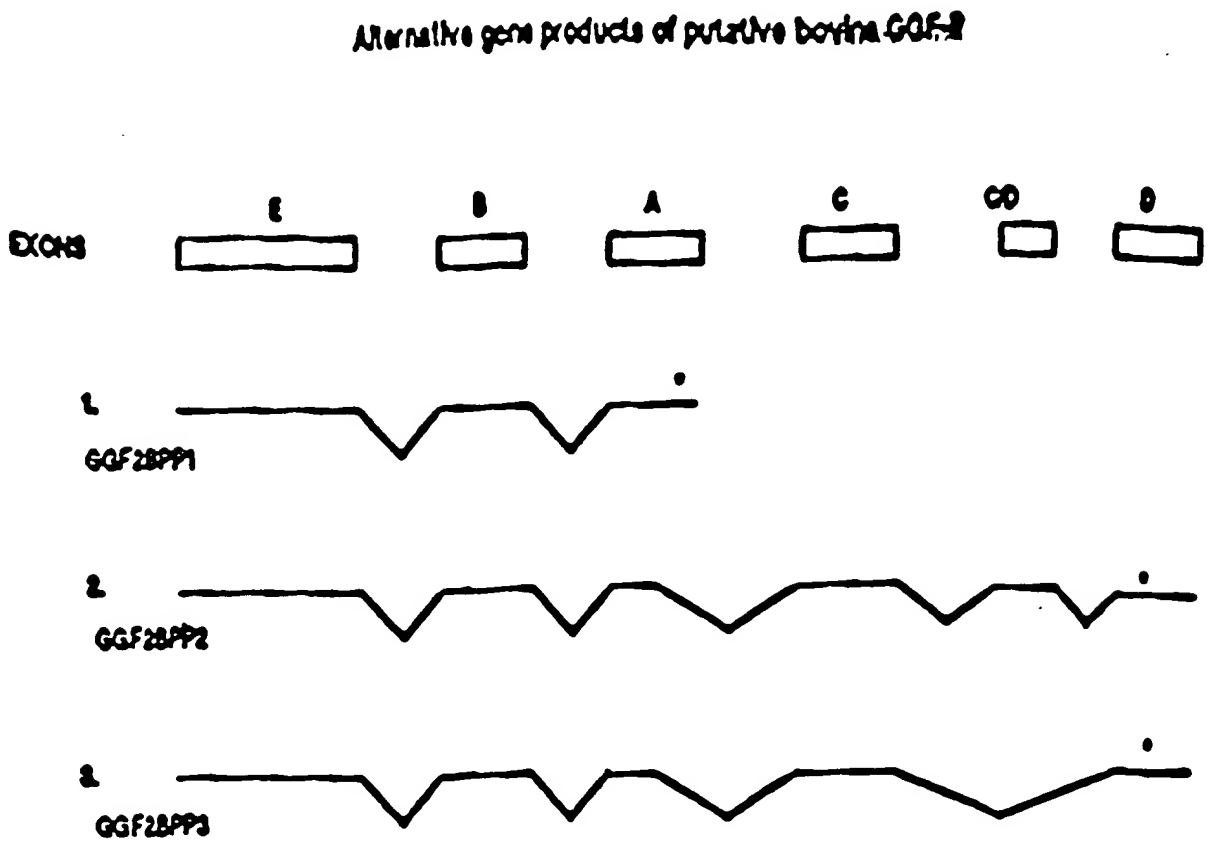
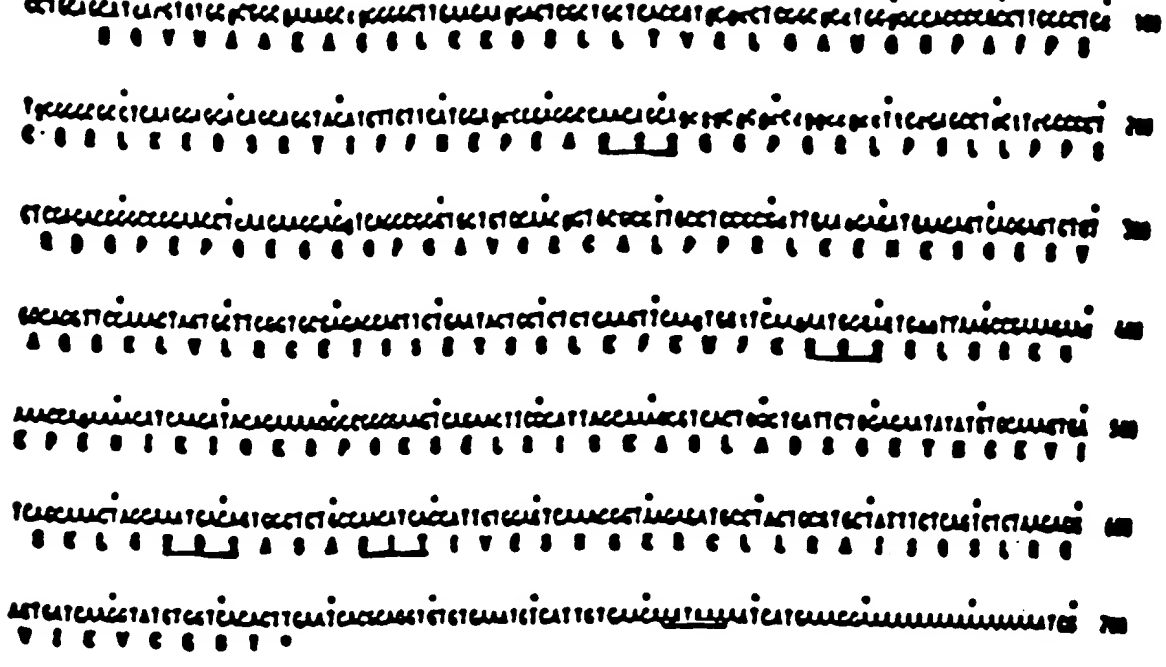

Alternative gene products of putative bovine GGF-2
EXONS
E
B
A
C
CO
D
1
GGF2BPP1
2
GGF2BPP2
3
GGF2BPP3

FIGURE 26

OCF-II peptide identified in deduced amino acid sequences of putative bovine OCF-II proteins

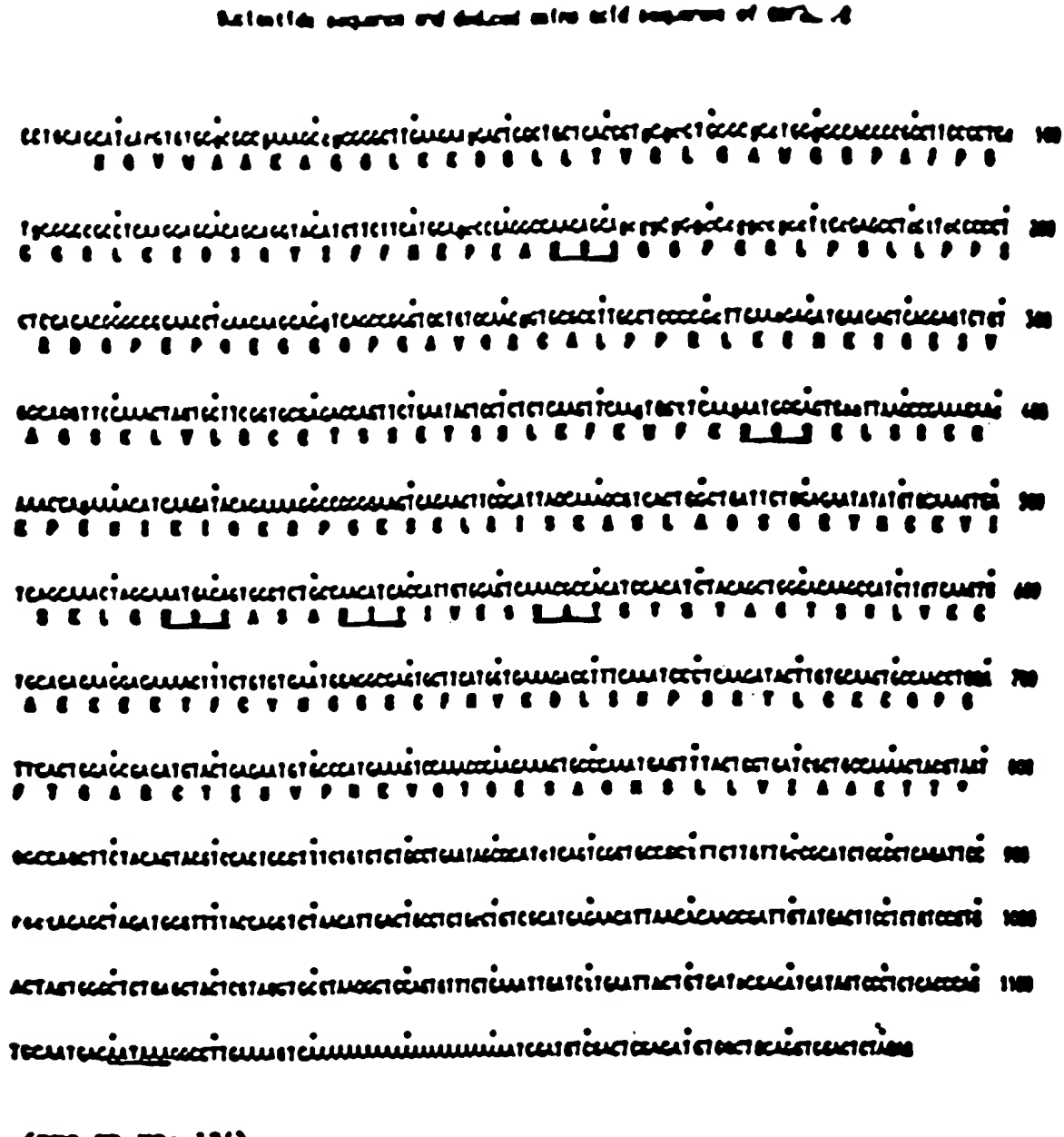
Peptide	Pos.	Sequence Match	
II-1		VHQVWAAK	
	1:	HQVWAAK AAGLK	(SEQ ID NO: 120)
II-10		DLLLXV	
	14:	GGLXX dslltv RLGAW	(SEQ ID NO: 121)
II-03		LGAWGPPAFPVXY	(SEQ ID NO: 122)
	21:	LLTVR lgavghpafpecq RLXED	(SEQ ID NO: 123)
II-02		YIFFXEPEAXSSG	(SEQ ID NO: 124)
	41:	XEDSR YIFFXEPEANSSG GPGRL	(SEQ ID NO: 125)
II-6		LVLR	
	103:	VAGSX LVLR CETSS	(SEQ ID NO: 126)
I-18		EYXCLXFFNTXXATVX	(SEQ ID NO: 127)
	112:	CETSS eysslxfxvfkngsel SRFXX	(SEQ ID NO: 128)
II-12		XASLADSGEYXXX	(SEQ ID NO: 129)
	151:	ELRIS XASLADSGEYXCX VISXL	(SEQ ID NO: 130)
I-07		ASLADEYEYXRX	(SEQ ID NO: 131)
	152:	LRISX asladsgeyack VISXL	(SEQ ID NO: 132)

FIGURE 27



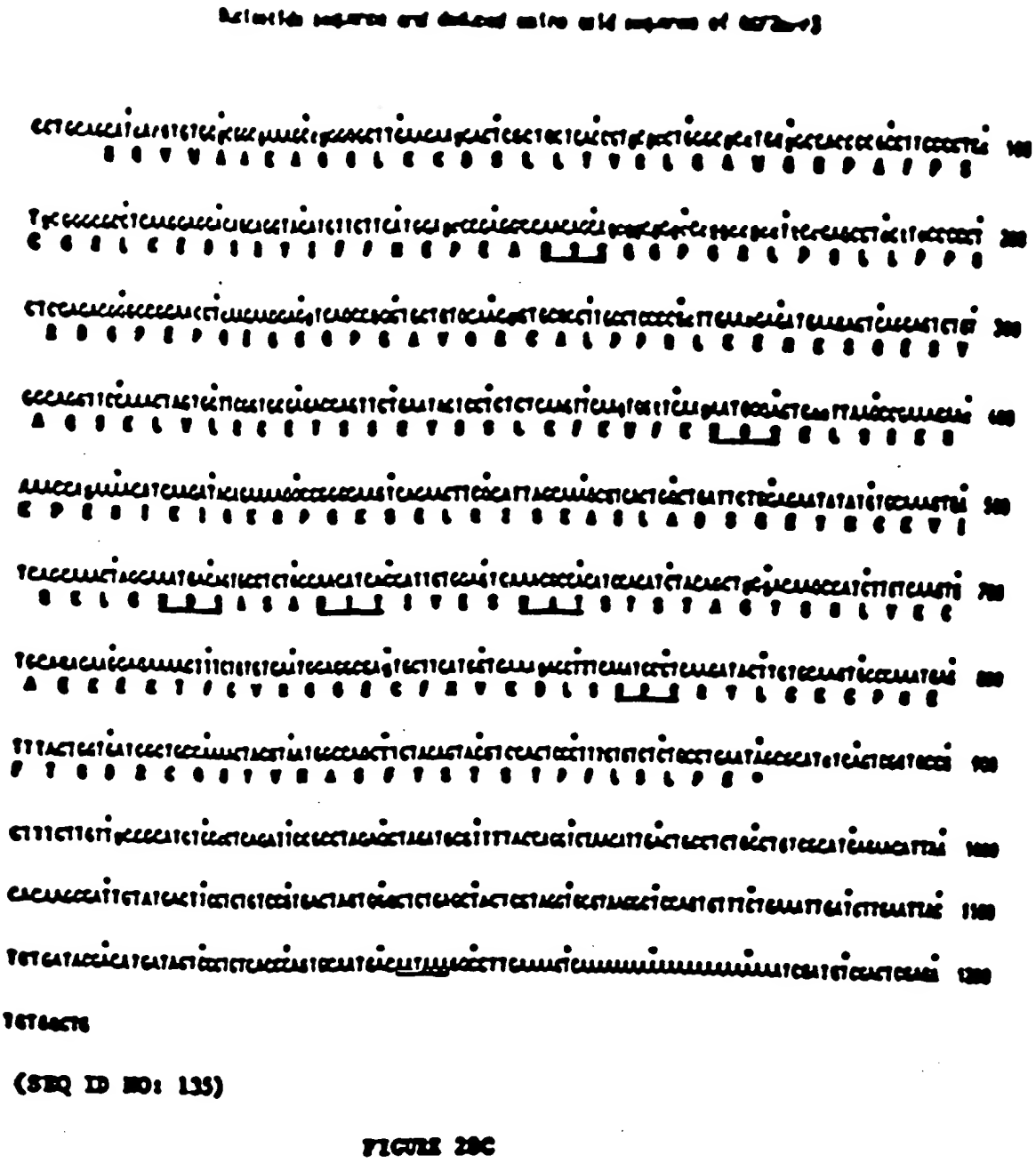
(SEQ ID NO: 133)

FIGURE 28A



(SEQ ID NO: 134)

FIGURE 28B



(SEQ ID NO: 135)

FIGURE 28C

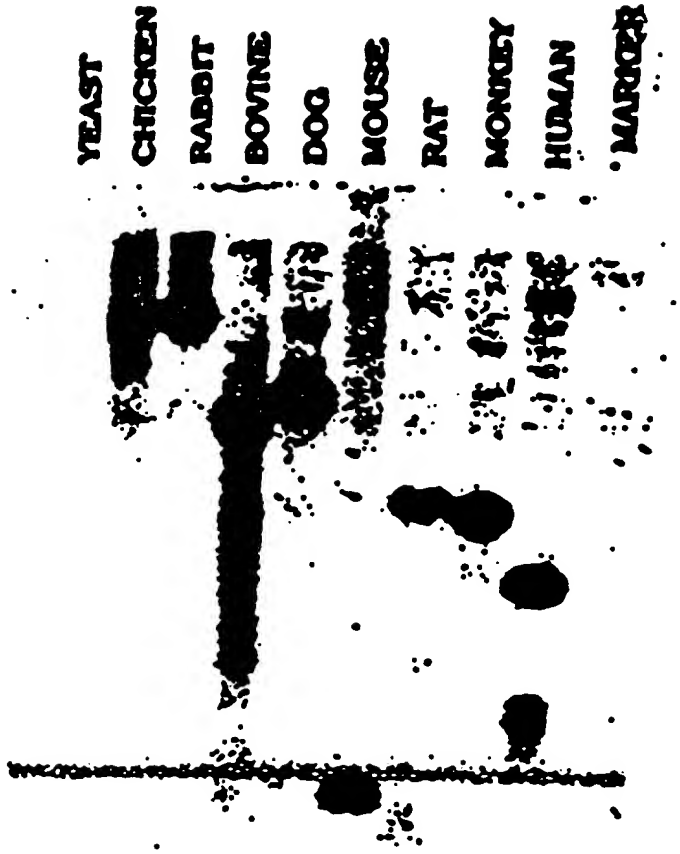

YEAST
CHICKEN
RABBIT
BOVINE
DOG
MOUSE
RAT
MONKEY
HUMAN
MARKER

FIGURE 29

FIGURE-30

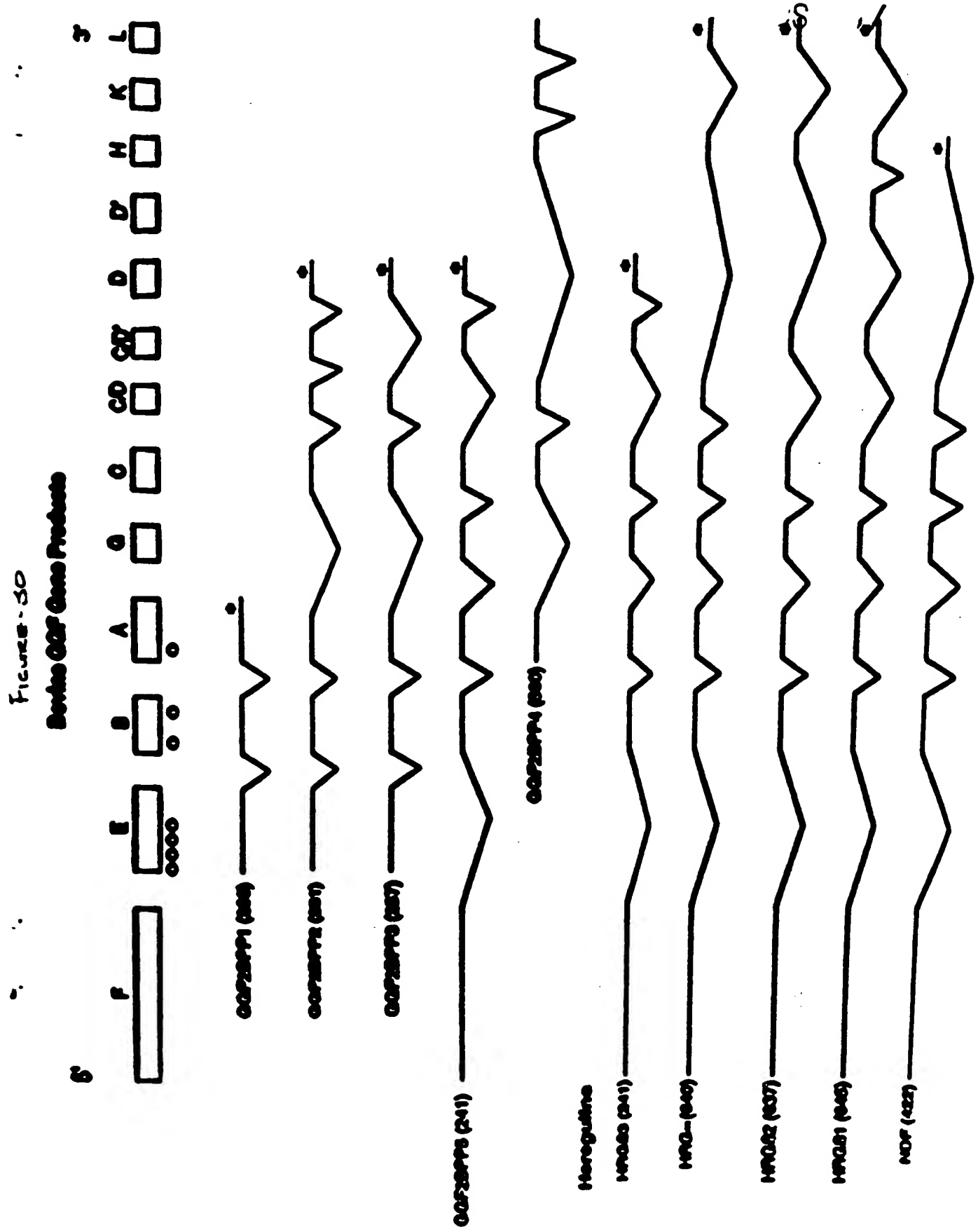
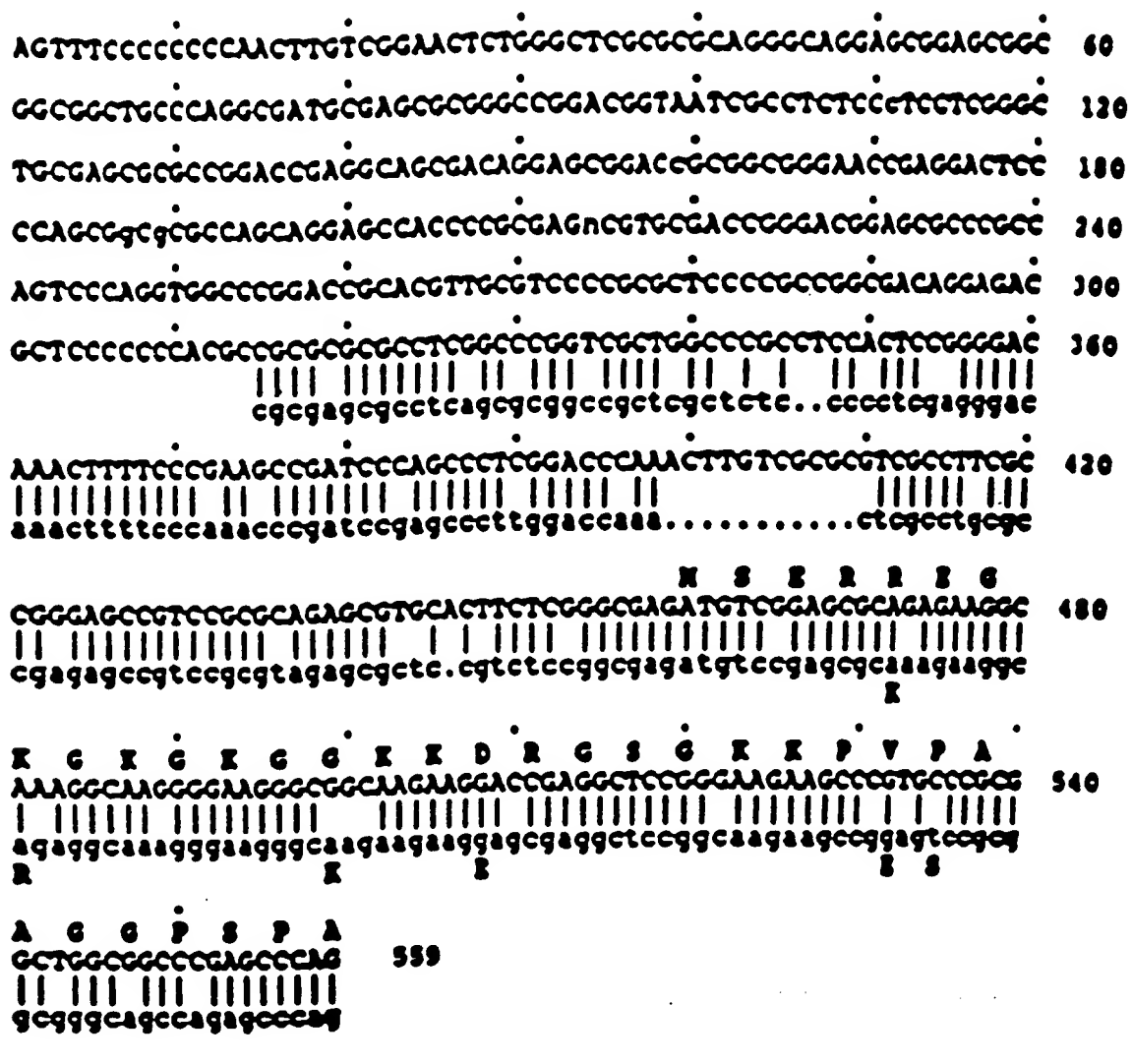


FIGURE 31 (1 of 8)

CODING SEGMENTS OF GLIAL GROWTH FACTOR/NEUREGULIN GENE

CODING SEGMENT F1 (SEQ ID NO: 136)



CODING SEGMENT E1 (SEQ ID NO: 137)

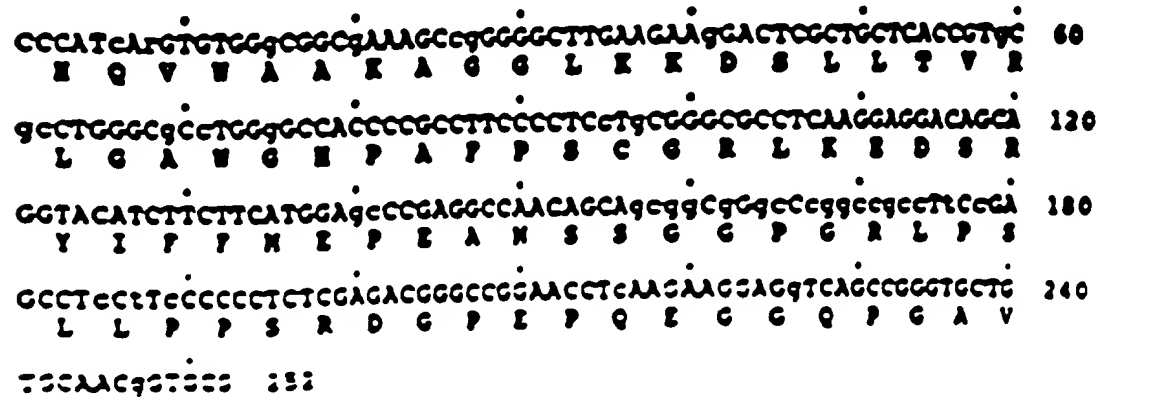
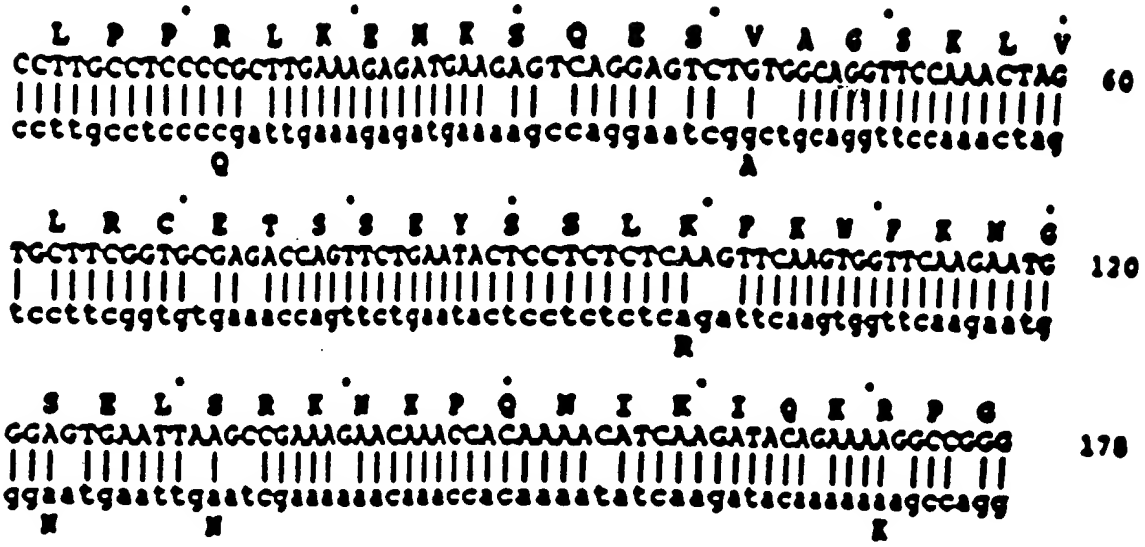
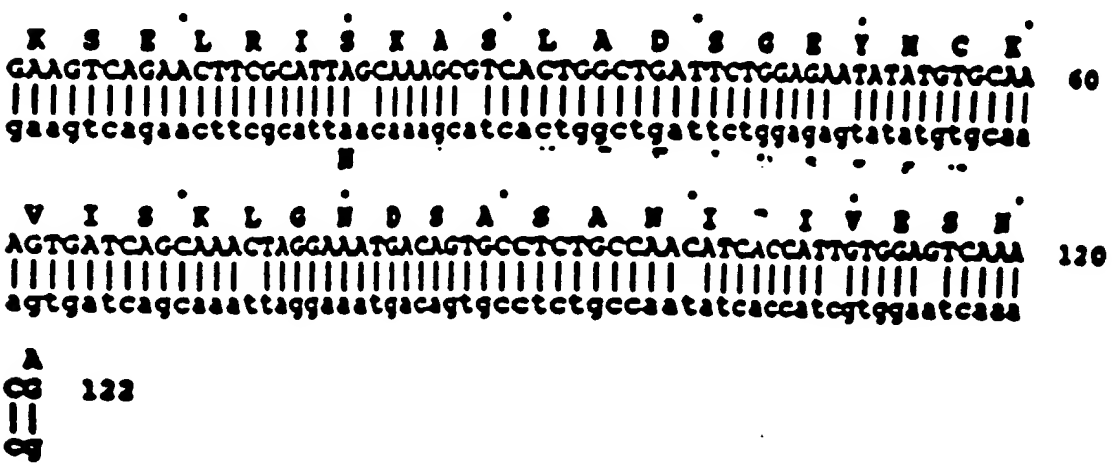


FIGURE 31 (CONT.)

CODING SEGMENT B: (SEQ ID NO: 138)



CODING SEGMENT A: (SEQ ID NO: 139)



CODING SEGMENT A': (SEQ ID NO: 140)

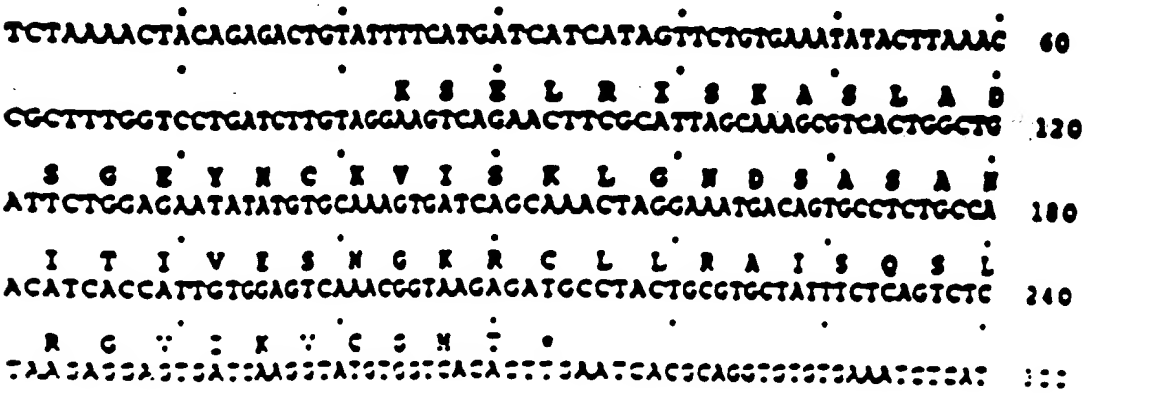


FIGURE 31 (CONT.) (3 8)

TGTGAACAAATAAAAATCATGAAAGGAAAACTCTATGTTTGAAATATCTTATGGGTCCTC 360

CTGTAAAGCTCTTCACTCCATAAGGTGAAATAGACCTGAAATATATATATAGATTATTT 417

CODING SEGMENT G: (SEQ ID NO: 141)

E I T T G N P A S T E T A Y V S S E S P I

AGATCACCACTGGCATGCCAGCCTCAACTGAGACAGCGTATGTGTCTTCAGAGTCTCCCA 60

agatcatcactggtatgccagcctcaactgaaggagcatatgtgtcttcagagtctccca

I G

R I S V S T E G T N T S S S

TTAGAATATCAGTATCCACAGAAGGAGCAAATACTTCTTCAT 102

ttagaatatcagtatccacagaaggagcaaatacttcttcat

A

CODING SEGMENT C: (SEQ ID NO: 160)

T S T S T A G T S N L V K C A E K E K T

CCACATCCACATCTACAGCTGGGACAAGCCATCTTGTCAAGTGTGCAGAGAAGGAGAAAA 60

ctacatctacatccaccactgggacaagccatcttgtaaaatgtgcggagaaggagaaaa

T

F C V N G G E C F N V K D L S N P S R I

CTTTCTGTGTGAATGGAGGCGAGTGCTTCATGGTGAAAGACCTTTCAAATCCCTCAAGAT 120

ctttctgtgtgaatggaggggagtgcttcatggtgaaagacctttcaaatccctcgagat

L C

ACTTGTGC 128

acttgtgc

FIGURE 31 (CONT.) (7 of 8)

CODING SEGMENT C/D: (SEQ ID NO: 142)

```
K  C  Q  P  G  F  T  G  A  R  C  T  E  M  V  P  M  K  V  Q
AAGTGCCAACCTGGATTCACTGGAGCGAGATGTACTGAGAATGTGCCCATGAAAGTCCAA  60
|||||||||||||||||||||||||| |||||||||||||||||||||||||||||||||
aagtgccaacctggattcactggagcgagatgtactgagaatgtgcccatgaaagtccaa

T  Q  E
ACCCAAGAA  69
|  |||||||
aaccaagaa
N
```

CODING SEGMENT C/D': (SEQ ID NO: 143)

```
K  C  P  N  E  F  T  G  D  R  C  Q  M  Y  V  M  A  S  F  Y
AAGTGCCCAAATGAGTTTACTGGTGATCGCTGCCAAAACTACGTAATGGCCAGCTTCTAC  60
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
aagtgcccaaatgagtttactggtgatcgctgccaaaactacgtaatggccagcttctac
```

CODING SEGMENT D: (SEQ ID NO: 144)

```
S  T  S  T  P  F  L  S  L  P  E  *
AGTACGTCCACTCCCTTTCTGTCTCTGCCTGAATAG  36
||||||||||||||||||||||||||||||||||||
agtacgtccactcccttctgtctctgcctgaatag
```

CODING SEGMENT D': (SEQ ID NO: 145)

```
K  M  L  G  I  E  F  M  E
aagcatcttgggattgaatttatggag  27
```

FIGURE 31 (CONT.) 5 -- 8)

CODING SEGMENT #1: (SEQ ID NO: 146)

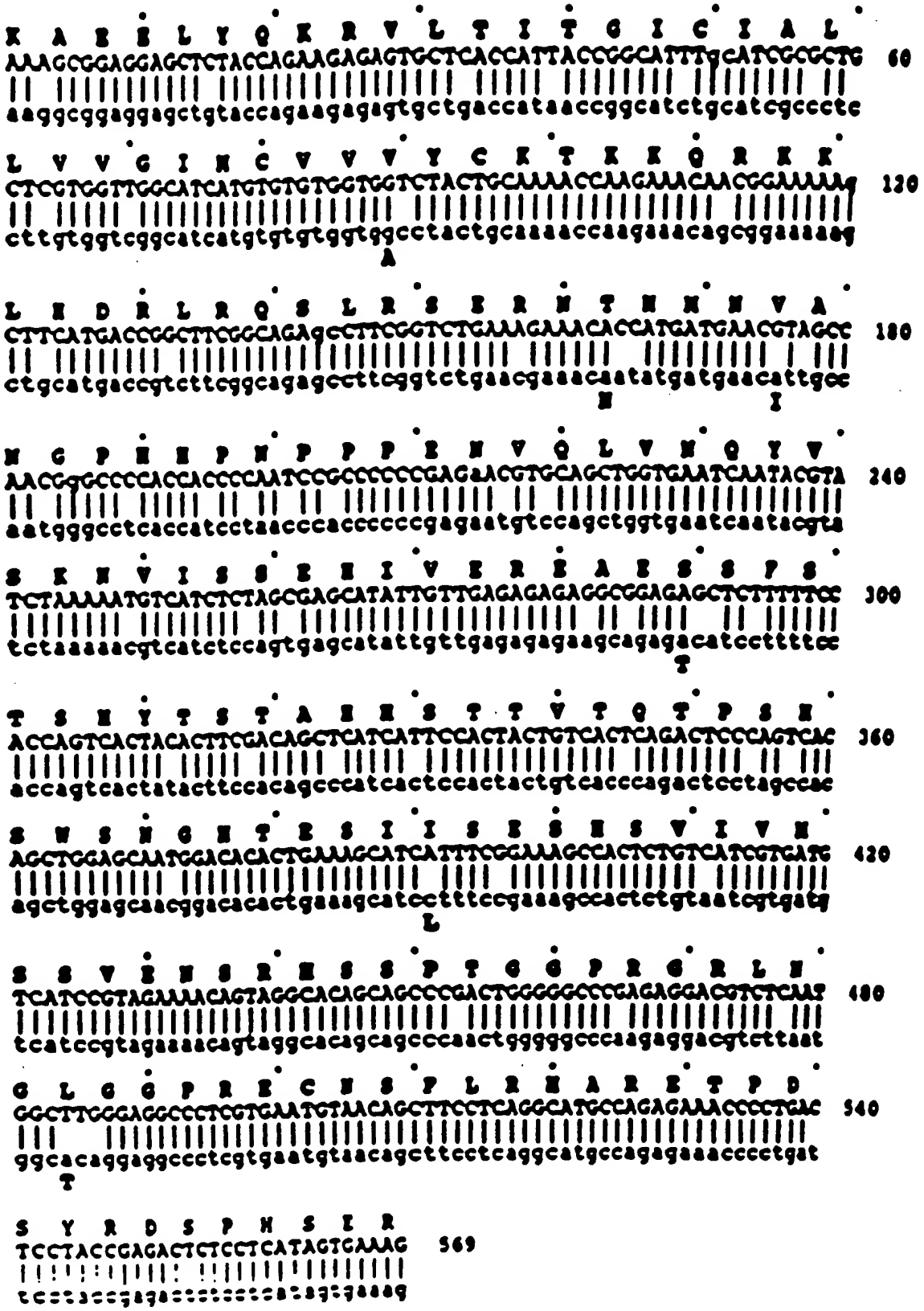
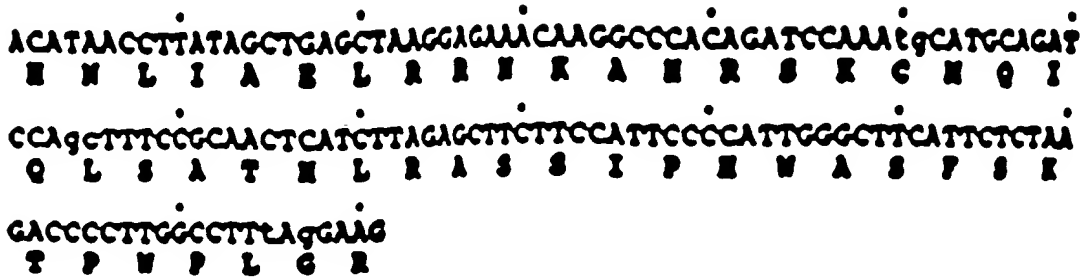


FIGURE 31 (CONT.)

CODING SEGMENT K: (SEQ ID NO: 161)



CODING SEGMENT L: (SEQ ID NO: 147)

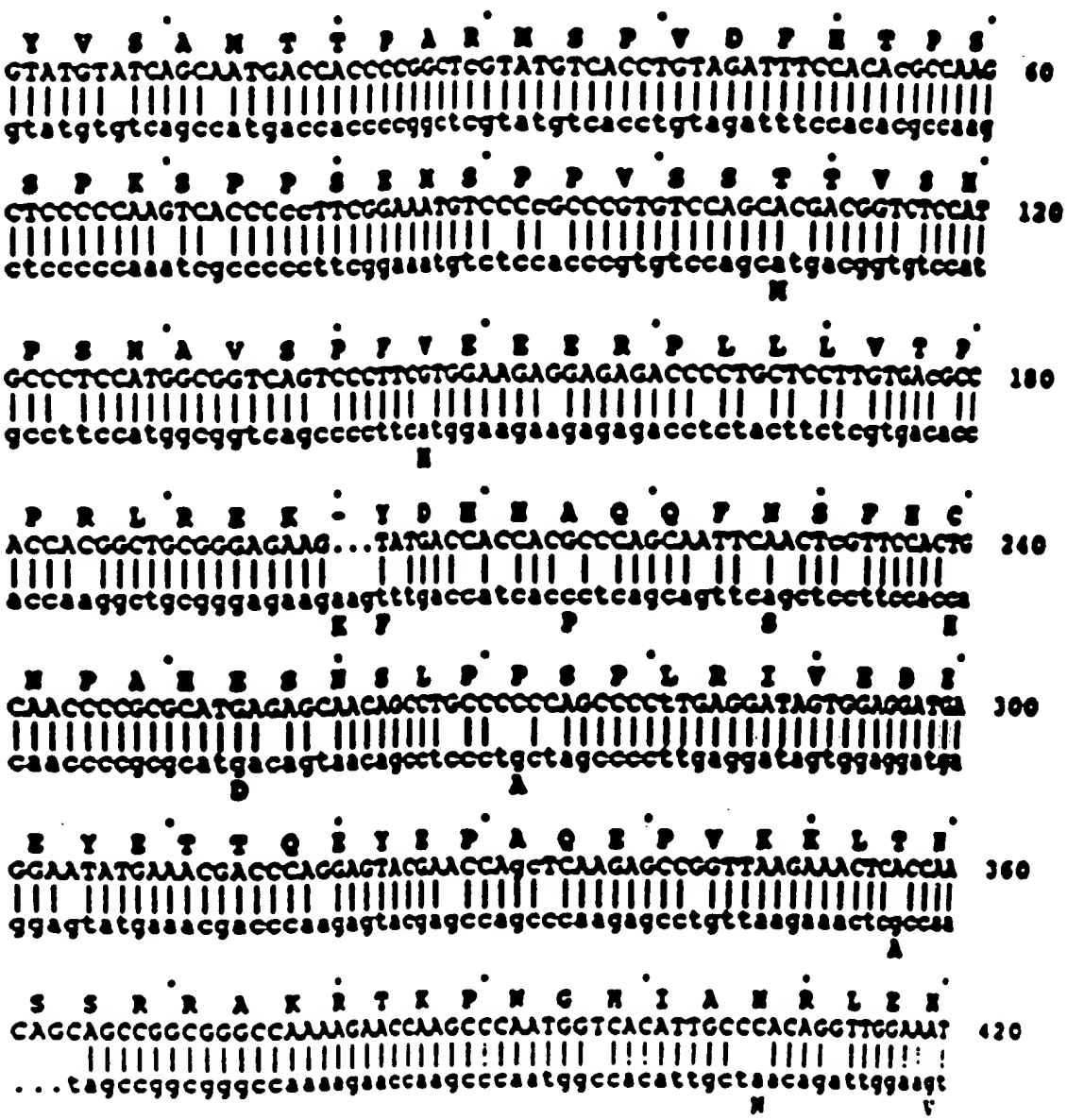


FIGURE 31 (CONT.) (SHEET 8)

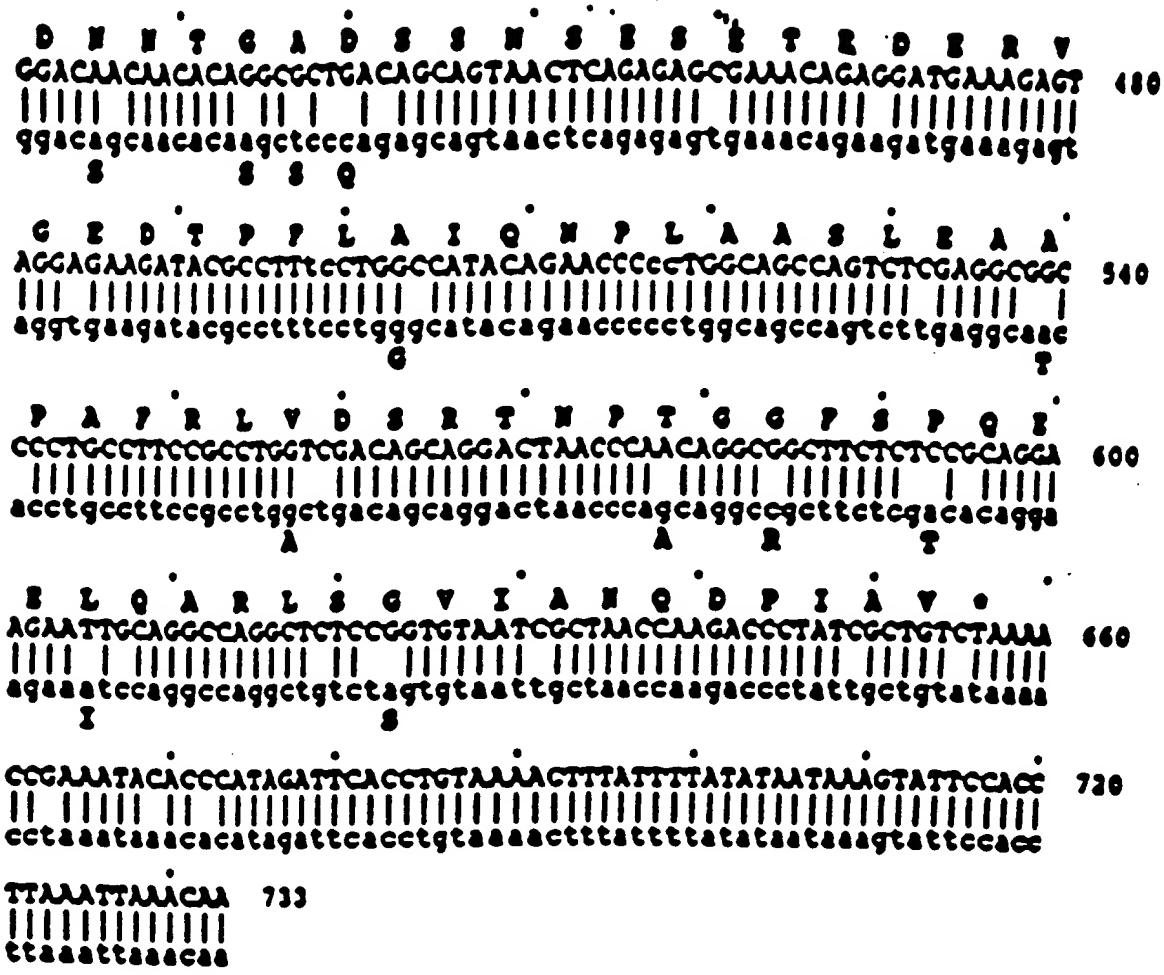


FIGURE 31 (CONT.) (8 of 8)

Human Coding Segment E: (SEQ ID NO: 163)

```
ATGAGATGGCGACGCGCCCCcgCgcCCCTCCGGGCGTCCCGGCCCCCGGGCCCAGCGCCCC   60
M  R  W  R  R  A  P  R  R  S  G  R  P  G  P  R  A  Q  R  P

GGCTCCGCCGCCCGCTCGTCGCCGCCGCTGCCGCTGCTGCCACTACTGCTGCTGCTGGGG  120
G  S  A  A  R  S  S  P  P  L  P  L  L  P  L  L  L  L  L  G

ACCGCGGCCCTGGCGCCGGGGCGGCGGCCGCAACGAGGCGGCTCCCGCGGGGCCTCG    180
T  A  A  L  A  P  G  A  A  A  G  N  E  A  A  P  A  G  A  S

GTGTGCTACTCGTCCCCGCCCAGCGTGGATCGGTGCAGGAGCTAGCTCAGCGCGCCGCG   240
V  C  Y  S  S  P  P  S  V  G  S  V  Q  E  L  A  Q  R  A  A

GTGGTGATCGAGGGAAAGGTGCACCCGCAGCGGCGGCAGCAGGGGCACTCGACAGGAAG  300
V  V  I  E  G  K  V  H  P  Q  R  R  Q  Q  G  A  L  D  R  K

GCGGCGGCGGCGGCGGCGGCAGGCAGGGGCGTGGGCGGCGATCGCCAGCCGCCAGCCGCG  360
A  A  A  A  A  G  E  A  G  A  W  G  G  D  R  E  P  P  A  A

GGCCCACGGGCGCTGGGGCCGCCCGCCGAGGAGCCGCTGCTCGCCGCCAACGGGACCGTG  420
G  P  R  A  L  G  P  P  A  E  E  P  L  L  A  A  N  G  T  V

CCCTCTTGGCCCACCGCCCCGGTGCCCAGCGCCGGCGAGCCCGGGGAGGAGGCGCCCTAT  480
P  S  W  P  T  A  P  V  P  S  A  G  E  P  G  E  E  A  P  Y

CTGGTGAAGGTGCACCAGGTGTGGGCGGTGAAAGCCGGGGGCTTGAAGAAGGACTCGCTG  540
L  V  K  V  H  Q  V  W  A  V  K  A  G  G  L  K  K  D  S  L

CTCACCGTGCGCCTGGGGACCTGGGGCCACCCCGCCTTCCCCTCCTGCGGGAGGCTCAAG  600
L  T  V  R  L  G  T  W  G  E  P  A  F  P  S  C  G  R  L  K

GAGGACAGCAGGTACATCTTCTTCATGGAGCCCGACGCCAACAGCACCAGCCGCGCCCG   660
E  D  S  R  Y  I  F  F  M  E  P  D  A  N  S  T  S  R  A  P

GCCGCCTTCCGAGCCTCTTTCCCCCCTCTGGAGACGGCCCGGAACCTCAAGAAGGAGGTC  720
A  A  F  R  A  S  F  P  P  L  E  T  G  R  N  L  K  K  E  V

AGCCGGGTGCTGTGCAAGCGGTGCC                                     749
S  R  V  L  C  K  R  C
```

HUMAN GCP CODING SEGMENT A'

```
GAAGTCAGAACTTCGCATTAACAAAGCCATCACTGCCTGATTCTGGAGAGTATATGTGCAA   60
 K  S  E  L  R  I  N  K  A  S  L  A  D  S  G  E  Y  M  C  K

AGTGATCAGCAAATTAGGAAATCACAGTGCCTCTGCCAATATCACCATCGTCGAATCAAA  120
 V  I  S  K  L  G  N  D  S  A  S  A  N  I  T  I  V  E  S  N

CGGTAAGAGATACCTACGGTATTCTGTTCCTCAATCTGTAACAAGAGTAATCAAAACATG  180
 G  K  R  Y  L  R  Y  S  V  P  Q  S  V  T  R  V  I  K  T  C

TGGTAAGACTCATAATAGACTGGTGTGTTAA  211
 G  K  T  H  N  R  L  V  C  *
```

(SEQ ID NO: 168)

(SEQ ID NO: 148) (1 .. 2)

FIGURE 32

GGF2BP5 nucleotide sequence and deduced protein sequence

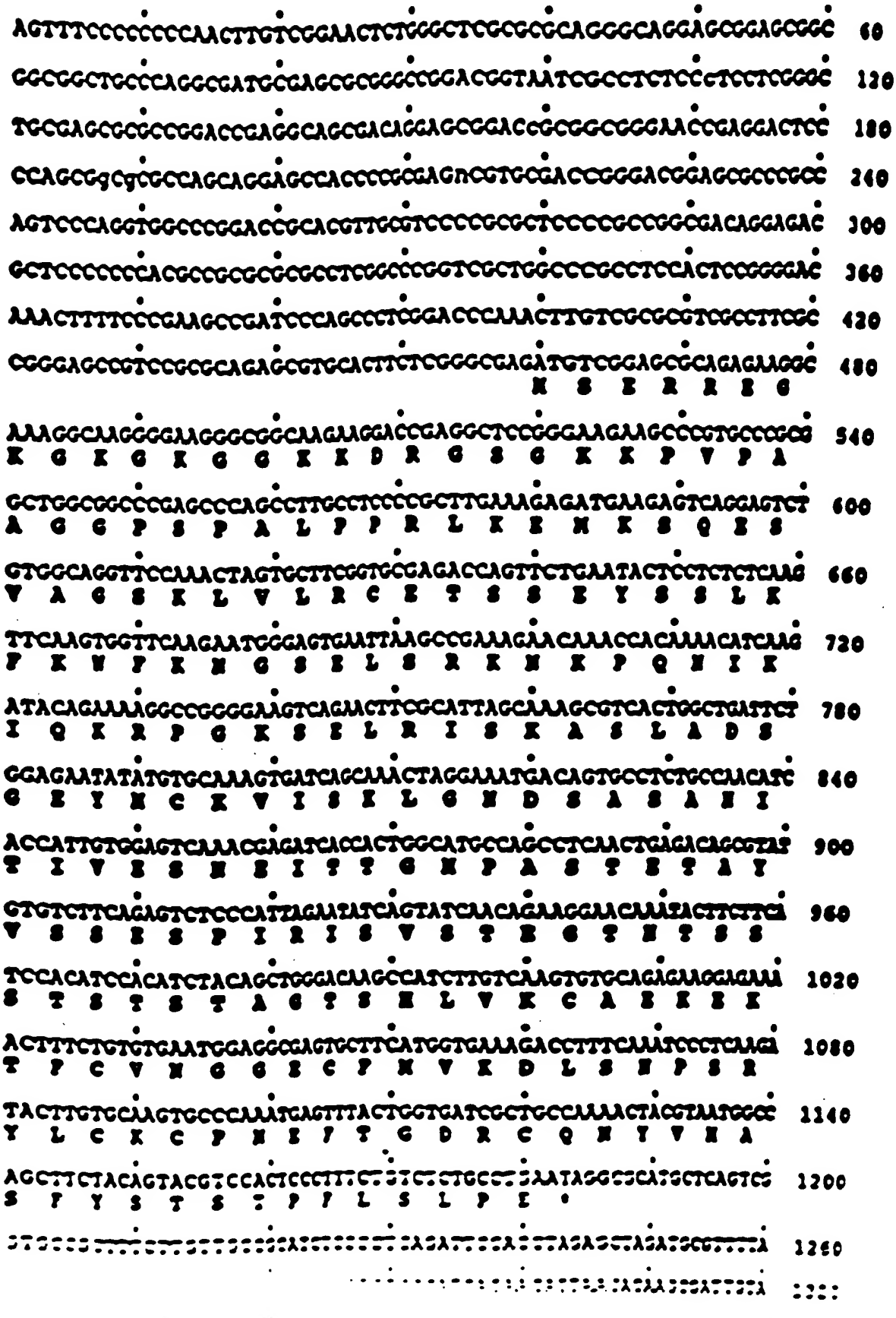


FIGURE 32 (CONT.) (2 of 2)

TGACTTCCTCTGTCCGTGACTAGTGGGCTCTGAGCTACTCGTAGGTGCGTAAGGCTCCAG 1380

TGTTTCTGAAATTGATCTTGAATTACTGTGATACGACATGATAGTCCCTCTCACCCAGTG 1440

CAATGACAATAAAGGCCTTGAAAAGTCTCACTTTTATTGAGAAAATAAAAATCGTTCCAC 1500

GGGACAGTCCCTCTTCTTTATAAAAATGACCCTATCCTTGAAAAGGAGGTGTGTTAAGTTG 1560

TAACCAGTACACACTTGAAATGATGGTAAGTTCGCTTCGGTTCAGAATGTGTTCTTTCTG 1620

ACAAATAAACAGAATAAAAAAAAAAAAAAAAA 1653

(SEQ ID NO: 149) FIGURE 33

GGP2BPP2 nucleotide sequence and deduced protein sequence

```
CATCArGTGTGGgCCCCgAAAGCCgGGGGCTTGAAGAAgGACTCGCTGCTCACCGTgCgC   60
M  Q  V  W  A  A  K  A  G  G  L  K  K  D  S  L  L  T  V  R

CTGGCCgCCTGGgCCCACCCCGCCTTCCCCTCCTgCGGGCGCCTCAAGGAGGACAGCAGG  120
L  G  A  W  G  H  P  A  F  P  S  C  G  R  L  K  E  D  S  R

TACATCTTCTTCATGGAgCCCGAGGCCAACAGCAgcggcgGgCCCggCGCCttCCGAGC  180
Y  I  F  F  M  E  P  E  A  N  S  S  G  G  P  G  R  L  P  S

CTCCTTCCCCCCTCTCGAGACGGGCCGGAACCTCAAGAAGGAGgTCAGCCGGGTGCTGTG  240
L  L  P  P  S  R  D  G  P  E  P  Q  E  G  G  Q  P  G  A  V

CAACgGTGCGCCTTGCCTCCCCGCTTGAAgGAGATGAAGAGTCAGGAGTCTGTGGCAGGT  300
Q  R  C  A  L  P  P  R  L  K  E  M  K  S  Q  E  S  V  A  G

TCCAAACTAGTGCTTCGGTGCGAGACCAGTTCTGAATACTCCTCTCTCAAGTTCAAgTGG  360
S  K  L  V  L  R  C  E  T  S  S  E  Y  S  S  L  K  F  K  W

tTCAAgAATGGGAGTGaaTTAAGCCGAAAGAACAAACCAgAAAACATCAAGATACAGAAA  420
F  K  N  G  S  E  L  S  R  K  N  K  P  E  N  I  K  I  Q  K

AGGCCCGGGAAGTCAGAACTTCGCATTAGCAAAGCGTCACTGGCTGATTCTGGAGAATAT  480
R  P  G  K  S  E  L  R  I  S  K  A  S  L  A  D  S  G  E  Y

ATGTGCAAAGTGATCAGCAAACTAGGAAATGACAGTGCCTCTGCCAACATCACCATTGTG  540
M  C  K  V  I  S  K  L  G  N  D  S  A  S  A  N  I  T  I  V

GAGTCAAACGCCACATCCACATCTACAGCTGGGACAAGCCATCTTGTCAAGTGTGCAGAG  600
E  S  N  A  T  S  T  S  T  A  G  T  S  N  L  V  K  C  A  E

AAGGAGAAAACTTTCTGTGTGAATGGAGGCGAGTGCTTCATGGTGAAAGACCTTTCAAAT  660
K  E  K  T  F  C  V  N  G  G  E  C  F  M  V  K  D  L  S  N

CCCTCAAGATACTTGTGCAAGTGCCAACCTGGATTCACTGGAGCGAGATGTACTGAGAAT  720
P  S  R  Y  L  C  K  C  Q  P  G  F  T  G  A  R  C  T  E  N

GTGCCCATGAAAGTCCAAACCCAAGAAAAGTGCCCAAATGAGTTTACTGGTGATCGCTGC  780
V  P  M  K  V  Q  T  Q  E  K  C  P  N  E  F  T  G  D  R  C

CAAAACTACGTAATGGCCAGCTTCTACAGTACGTCCACTCCCTTTCTGTCTCTGCCTGAA  840
Q  N  Y  V  M  A  S  F  Y  S  T  S  T  P  F  L  S  L  P  E

TAGCGCATCTCAGTCGGTGCCGCTTTCTTGTTGCCGCATCTCCCCTCAGATTCCTCCTAG  900

AGCTAGATGCGTTTTACCAGGTCTAACATTGACTGCCTCTGCCTGTCGCATGAGAACATT  960

AACACAAGCGATTGTATGACTTCCTCTGTCCGTGACTAGTGGGCTCTGAGCTACTCGTAG  1020

GTGCGTAAGGCTCCAGTGTTTCTGAAATTGATCTTGAATTACTGTGATACGACATGATAG  1080

TCCCTCTCACCCAGTGCAATGACAATAAAGGCCTTGAAAGTCAAAAAAAAAAAAAAA  1140
```

(SEQ ID NO: 150) (1 of 2) FIGURE 34

GGF2BPP4 nucleotide sequence and deduced protein sequence

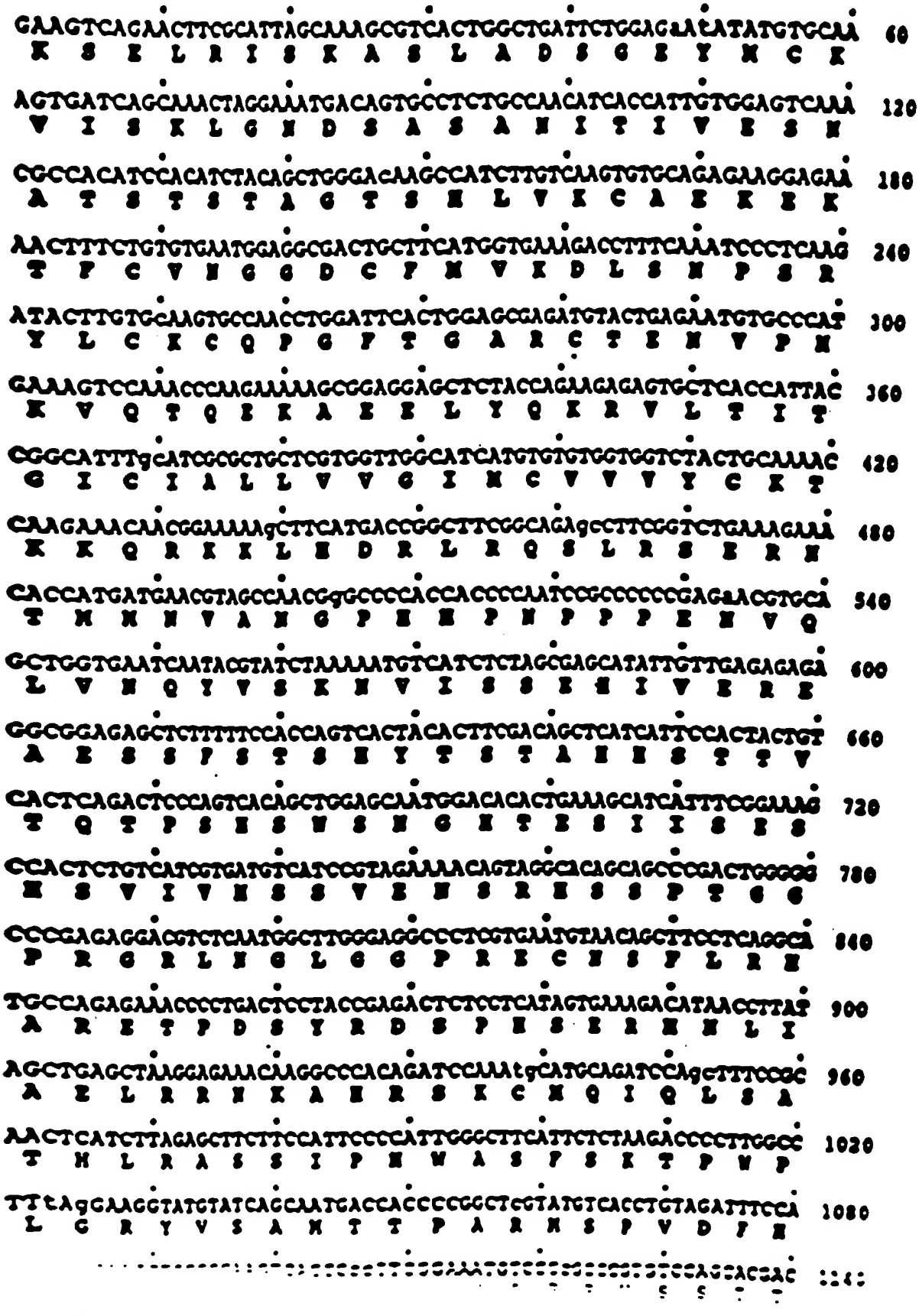


FIGURE 34 (CONT.) (2 of _,

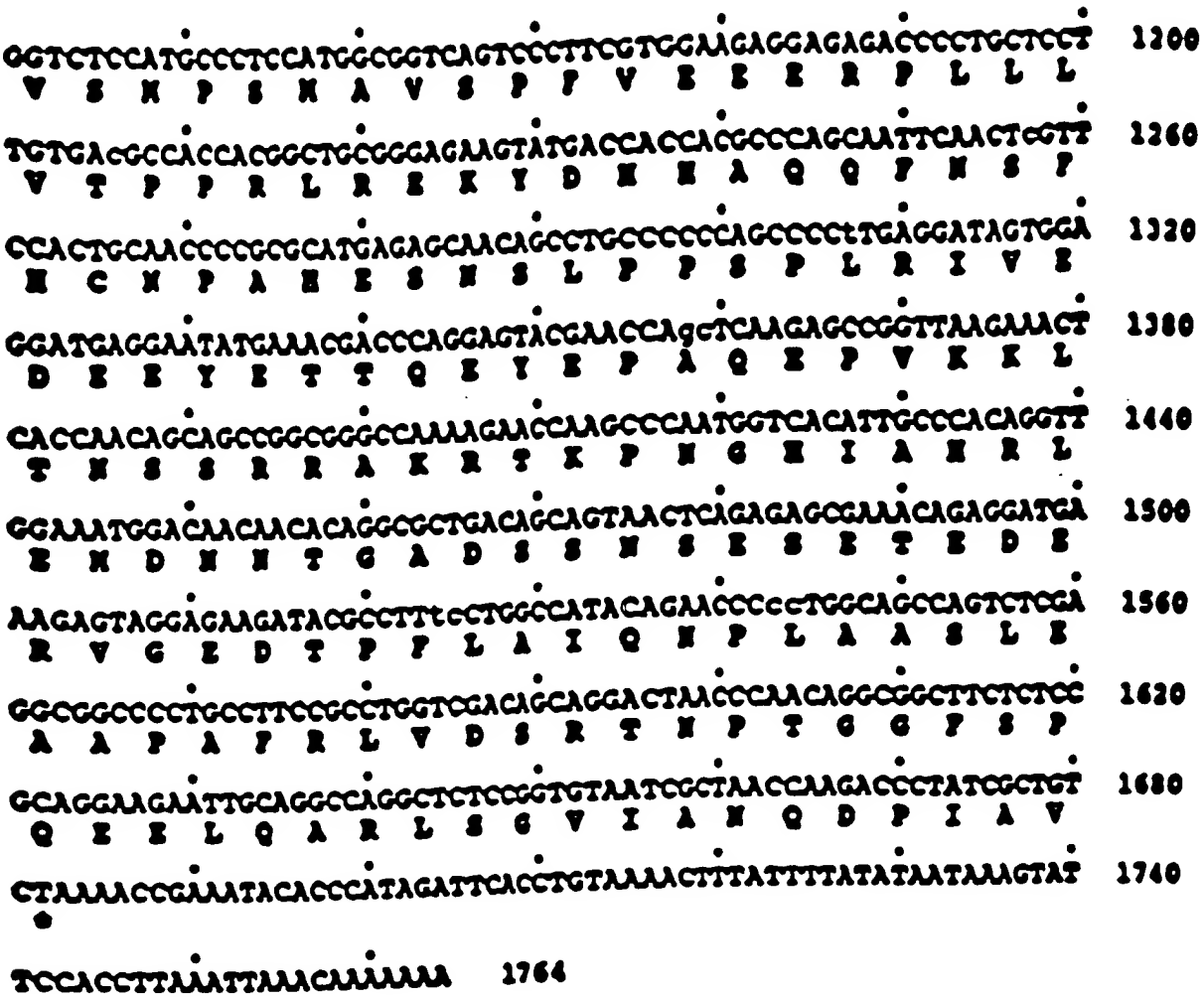


FIGURE 35

1(SEQ ID NO: 151)
2(SEQ ID NO: 152)
3(SEQ ID NOL 153)

FIGURE 36

200 kDa tyrosine phosphorylation compared with mitogenic activity

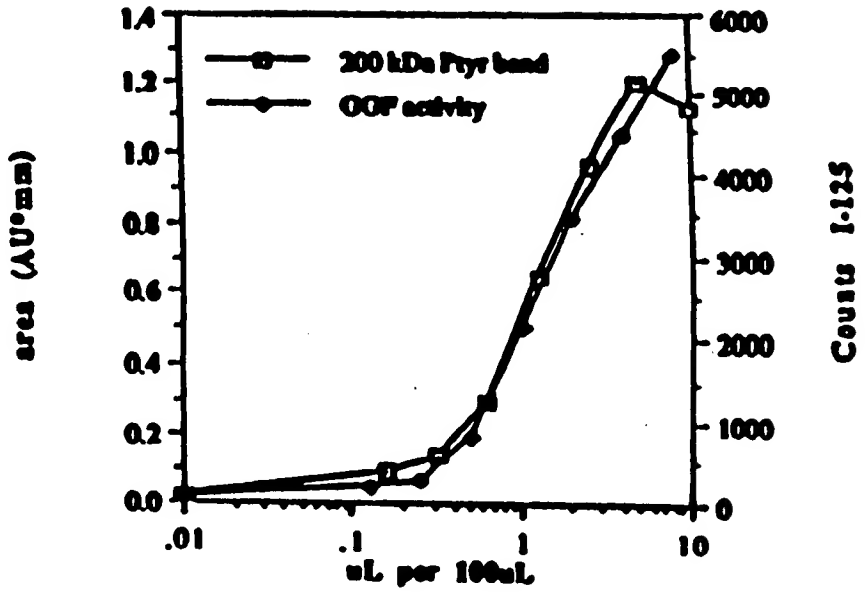


FIGURE 37 (1 of 2)

GGF/HEREGULIN SPLICING VARIANTS

F-B-A'

F-B-A-C-C/D-D
F-B-A-C-C/D-H
F-B-A-C-C/D-H-L
F-B-A-C-C/D-H-K-L
F-B-A-C-C/D-D'-H
F-B-A-C-C/D-D'-H-L
F-B-A-C-C/D-D'-H-K-L
F-B-A-C-C/D'-D
F-B-A-C-C/D'-H
F-B-A-C-C/D'-H-L
F-B-A-C-C/D'-H-K-L
F-B-A-C-C/D'-D'-H
F-B-A-C-C/D'-D'-H-L
F-B-A-C-C/D'-D'-H-K-L
F-B-A-C-C/D-C/D'-D
F-B-A-C-C/D-C/D'-H
F-B-A-C-C/D-C/D'-H-L
F-B-A-C-C/D-C/D'-H-K-L
F-B-A-C-C/D-C/D'-D'-H
F-B-A-C-C/D-C/D'-D'-H-L
F-B-A-C-C/D-C/D'-D'-H-K-L

F-E-B-A'

F-E-B-A-C-C/D-D
F-E-B-A-C-C/D-H
F-E-B-A-C-C/D-H-L
F-E-B-A-C-C/D-H-K-L
F-E-B-A-C-C/D-D'-H
F-E-B-A-C-C/D-D'-H-L
F-E-B-A-C-C/D-D'-H-K-L
F-E-B-A-C-C/D'-D
F-E-B-A-C-C/D'-H
F-E-B-A-C-C/D'-H-L
F-E-B-A-C-C/D'-H-K-L
F-E-B-A-C-C/D'-D'-H
F-E-B-A-C-C/D'-D'-H-L
F-E-B-A-C-C/D'-D'-H-K-L
F-E-B-A-C-C/D-C/D'-D
F-E-B-A-C-C/D-C/D'-H
F-E-B-A-C-C/D-C/D'-H-L
F-E-B-A-C-C/D-C/D'-H-K-L
F-E-B-A-C-C/D-C/D'-D'-H
F-E-B-A-C-C/D-C/D'-D'-H-L
F-E-B-A-C-C/D-C/D'-D'-H-K-L

F-B-A-G-C-C/D-D
F-B-A-G-C-C/D-H
F-B-A-G-C-C/D-H-L
F-B-A-G-C-C/D-H-K-L
F-B-A-G-C-C/D-D'-H
F-B-A-G-C-C/D-D'-H-L
F-B-A-G-C-C/D-D'-H-K-L
F-B-A-G-C-C/D'-D
F-B-A-G-C-C/D'-H
F-B-A-G-C-C/D'-H-L
F-B-A-G-C-C/D'-H-K-L
F-B-A-G-C-C/D'-D'-H
F-B-A-G-C-C/D'-D'-H-L
F-B-A-G-C-C/D'-D'-H-K-L
F-B-A-G-C-C/D-C/D'-D
F-B-A-G-C-C/D-C/D'-H
F-B-A-G-C-C/D-C/D'-H-L
F-B-A-G-C-C/D-C/D'-H-K-L
F-B-A-G-C-C/D-C/D'-D'-H
F-B-A-G-C-C/D-C/D'-D'-H-L
F-B-A-G-C-C/D-C/D'-D'-H-K-L

F-E-B-A-G-C-C/D-D
F-E-B-A-G-C-C/D-H
F-E-B-A-G-C-C/D-H-L
F-E-B-A-G-C-C/D-H-K-L
F-E-B-A-G-C-C/D-D'-H
F-E-B-A-G-C-C/D-D'-H-L
F-E-B-A-G-C-C/D-D'-H-K-L
F-E-B-A-G-C-C/D'-D
F-E-B-A-G-C-C/D'-H
F-E-B-A-G-C-C/D'-H-L
F-E-B-A-G-C-C/D'-H-K-L
F-E-B-A-G-C-C/D'-D'-H
F-E-B-A-G-C-C/D'-D'-H-L
F-E-B-A-G-C-C/D'-D'-H-K-L
F-E-B-A-G-C-C/D-C/D'-D
F-E-B-A-G-C-C/D-C/D'-H
F-E-B-A-G-C-C/D-C/D'-H-L
F-E-B-A-G-C-C/D-C/D'-H-K-L
F-E-B-A-G-C-C/D-C/D'-D'-H
F-E-B-A-G-C-C/D-C/D'-D'-H-L
F-E-B-A-G-C-C/D-C/D'-D'-H-K-L

FIGURE 37 (CONT.) (2 of 2)

OGP/HERCULIN SPLICING VARIANTS CONTINUED

E-B-A'

E-B-A-C-C/D-D
E-B-A-C-C/D-H
E-B-A-C-C/D-H-L
E-B-A-C-C/D-H-K-L
E-B-A-C-C/D-D'-H
E-B-A-C-C/D-D'-H-L
E-B-A-C-C/D-D'-H-K-L
E-B-A-C-C/D'-D
E-B-A-C-C/D'-H
E-B-A-C-C/D'-H-L
E-B-A-C-C/D'-H-K-L
E-B-A-C-C/D'-D'-H
E-B-A-C-C/D'-D'-H-L
E-B-A-C-C/D'-D'-H-K-L
E-B-A-C-C/D-C/D'-D
E-B-A-C-C/D-C/D'-H
E-B-A-C-C/D-C/D'-H-L
E-B-A-C-C/D-C/D'-H-K-L
E-B-A-C-C/D-C/D'-D'-H
E-B-A-C-C/D-C/D'-D'-H-L
E-B-A-C-C/D-C/D'-D'-H-K-L

E-B-A-G-C-C/D-D
E-B-A-G-C-C/D-H
E-B-A-G-C-C/D-H-L
E-B-A-G-C-C/D-H-K-L
E-B-A-G-C-C/D-D'-H
E-B-A-G-C-C/D-D'-H-L
E-B-A-G-C-C/D-D'-H-K-L
E-B-A-G-C-C/D'-D
E-B-A-G-C-C/D'-H
E-B-A-G-C-C/D'-H-L
E-B-A-G-C-C/D'-H-K-L
E-B-A-G-C-C/D'-D'-H
E-B-A-G-C-C/D'-D'-H-L
E-B-A-G-C-C/D'-D'-H-K-L
E-B-A-G-C-C/D-C/D'-D
E-B-A-G-C-C/D-C/D'-H
E-B-A-G-C-C/D-C/D'-H-L
E-B-A-G-C-C/D-C/D'-H-K-L
E-B-A-G-C-C/D-C/D'-D'-H
E-B-A-G-C-C/D-C/D'-D'-H-L
E-B-A-G-C-C/D-C/D'-D'-H-K-L

FIGURE 38

EGFL1

AGCCATCTTGTCAAGTGTGCAGAGAAGGAGAAAACTTTCTGTGTGAATGGAGGCGAGTGC
S H L V K C A E K E K T F C V N G G E C

TTCATGGTGAAAGACCTTTCAAATCCCTCAAGATACTTGTGCAAGTGCCCAAATGAGTTT
F M V K D L S N P S R Y L C K C P N E F

ACTGGTGATCGCTGCCAAAACTACGTAATGGCCAGCTTCTACAGTACGTCCACTCCCTTT
T G D R C Q N Y V M A S F Y S T S T P F

CTGTCTCTGCCTGAATAG
L S L P E *

FIGURE 39

(SEQ ID NO: 154)

EGFL2

AGCCATCTTGTCAAGTGTGCAGAGAAGGAGAAAACTTTCTGTGTGAATGGAGGCGAGTGC
S H L V K C A E K E K T F C V N G G E C

TTCATGGTGAAAGACCTTTCAAATCCCTCAAGATACTTGTGCAAGTGCCAACCTGGATTC
F M V K D L S N P S R Y L C K C Q P G F

ACTGGAGCGAGATGTACTGAGAATGTGCCCATGAAAGTCCAAACCCAAGAAAAAGCCGAG
T G A R C T E N V P M K V Q T Q E K A E

GAGCTCTACTAA
E L Y *

FIGURE 40

(SEQ ID NO: 155)

EGFL3

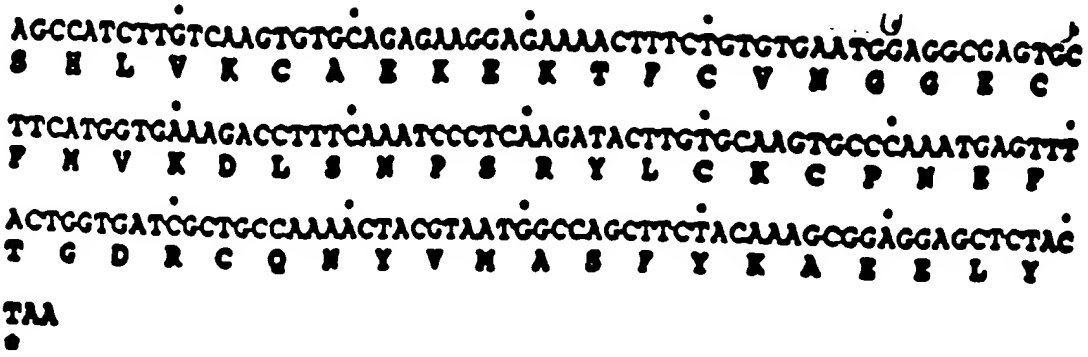


FIGURE 41

(SEQ ID NO: 156)

EGFLA

AGCCATCTTGTCAAGTGTGCAGAGAAGGAGAAAACTTTCTGTGTGAATGGAGGCGAGTGC
S H L V K C A E K E K T F C V N G G E C

TTCATGGTGAAAGACCTTTCAAATCCCTCAAGATACTTGTGCAAGTGCCCAAATCAGTTT
F M V K D L S N P S R Y L C K C P N E F

ACTGGTGATCGCTGCCAAAACTACGTAATGGCCAGCTTCTACAAGCATCTTGGGATTGAA
T G D R C Q N Y V M A S F Y K H L G I E

TTTATGGAGAAAGCGGAGGAGCTCTACTAA
F M E K A E E L Y *

FIGURE 42

(SEQ ID NO: 157)

EGFLS

AGCCATCTTGTCAAGTGTGCAGAGAAGGAGAAAACTTTCTGTGTGAATGGAGGCGAGTGC
S H L V K C A E K E K T F C V N G G E C

TTCATGGTGAAAGACCTTTCAAATCCCTCAAGATACTTGTGCAAGTGCCAACCTGGATTC
F M V K D L S N P S R Y L C K C Q P G F

ACTGGAGCGAGATGTACTGAGAATGTGCCCATGAAAGTCCAAACCCAAGAAAAGTGCCCA
T G A R C T E N V P M K V Q T Q E K C P

AATGAGTTTACTGGTGATCGCTGCCAAAACTACGTAATGGCCAGCTTCTACAGTACGTCC
N E F T G D R C Q N Y V M A S F Y S T S

ACTCCCTTTCTGTCTCTGCCTGAATAG
T P F L S L P E *

FIGURE 43

(SEQ ID NO: 158)

EGFL6

```
AGCCATCTTGTCAAGTGTGCAGAGAAGGAGAAAACTTTCTGTGTGAATGGAGGCGAGTGC
S  H  L  V  K  C  A  E  K  E  K  T  F  C  V  N  G  G  E  C

TTCATGGTGAAAGACCTTTCAAATCCCTCAAGATACTTGTGCCAGTGCCAACCTGGATTC
F  M  V  K  D  L  S  N  P  S  R  Y  L  C  K  C  Q  P  G  F

ACTGGAGCGAGATGTACTGAGAATGTGCCCATGAAAGTCCAAACCCAAGAAAAGTGCCCA
T  G  A  R  C  T  E  N  V  P  M  K  V  Q  T  Q  E  K  C  P

AATGAGTTTACTGGTGATCGCTGCCAAAACTACGTAATGGCCAGCTTCTACAAAGCGGAG
N  E  F  T  G  D  R  C  Q  N  Y  V  M  A  S  F  Y  K  A  E

GAGCTCTACTAA
E  L  Y  *
```

FIGURE 44

(SEQ ID NO: 159)

Nucleotide sequence and deduced amino acid sequence of GGF2HBS5

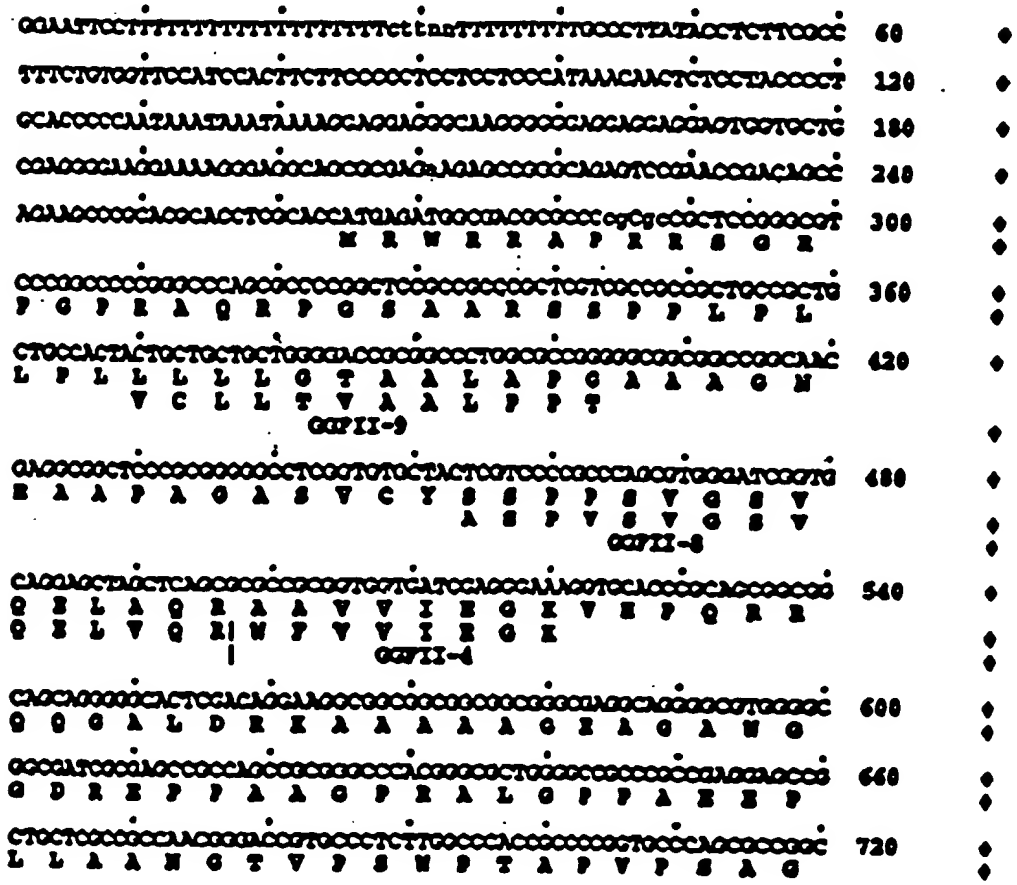


FIGURE 45 (1 of 3)

(SEQ ID NO: 167)

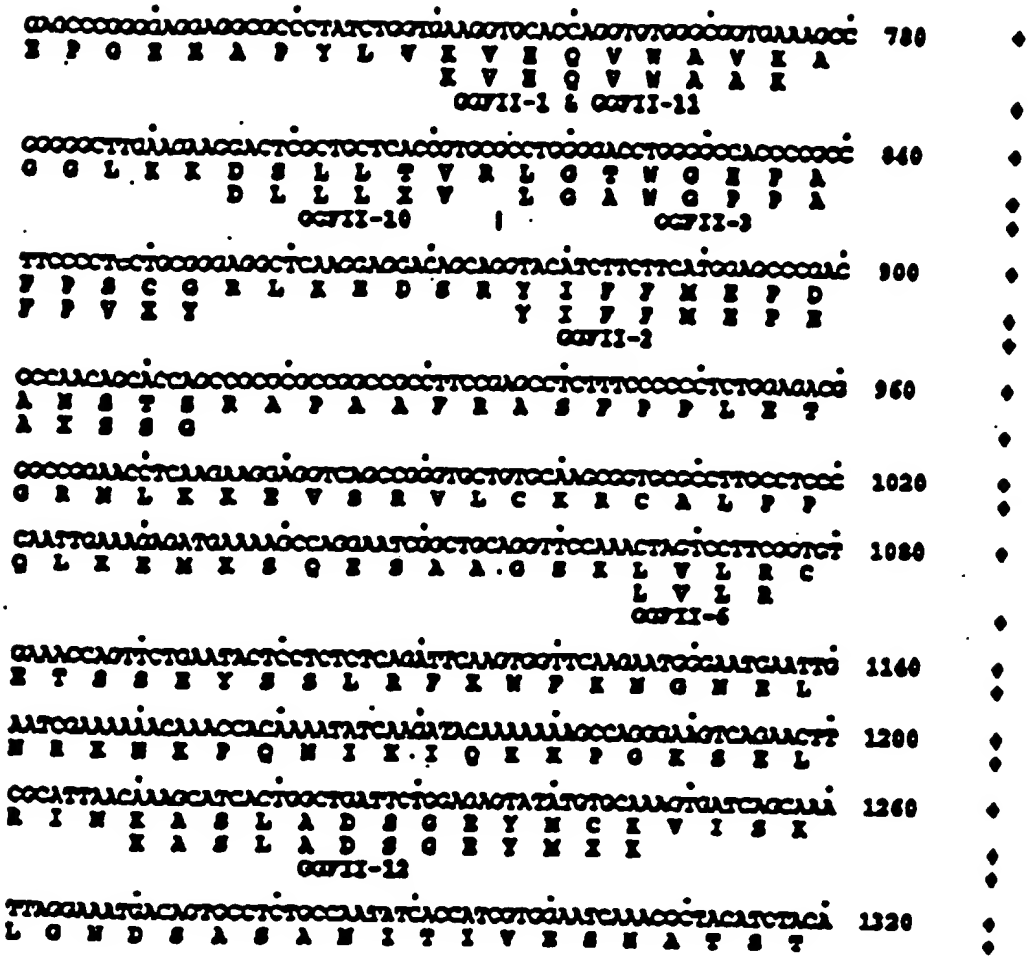


FIGURE 45 (2 of 3)

(SEQ ID NO: 167)

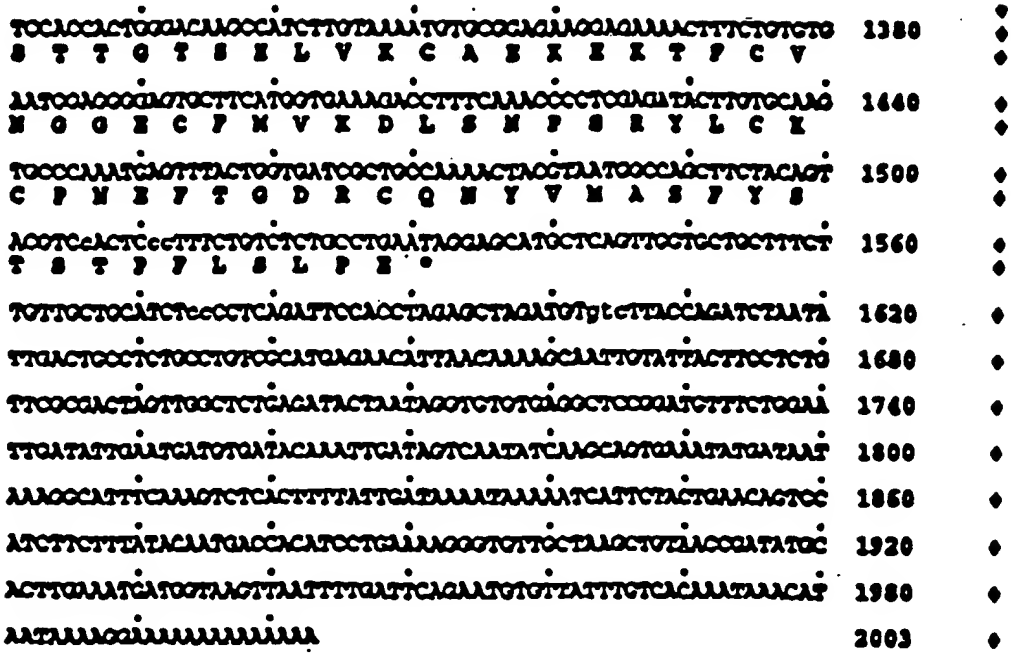


FIGURE 45 (3 of 3)

(SEQ ID NO: 167)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07491

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :435/6; 530/350; 536/22.1; 424/88
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 530/350; 536/22.1; 424/88

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NONE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
23 September 1993	12 OCT 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer DAVID R. PRESTON
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07491

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C12Q 1/68; C07K 3/00, 13/00, 15/00, 17/00; C07H 19/00, 21/00; A61K 39/00